Integration, persistence and control of hygienically relevant bacteria in iron oxide incrustations in wells

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"Every problem, […], is an opportunity in disguise."

I. Serra

"And it's brought me a galaxy of fun […]."

M. Reynolds

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Abstract

Groundwater can become contaminated by hygienically relevant microorganisms and pathogens, for example, in consequence of heavy rainstorms, flooding of wells or hydraulic short circuits between surface water and groundwater. In case of an entry of hygienically relevant microorganisms into the aquifer, or by direct ingress, these organisms can reach drinking-water wells. If incrustations like oxides or oxyhydroxides of Fe(III), which represent the most common incrustation type in Germany, exist in the water well, the microorganisms encounter a very large and porous surface to colonise. Therefore, it was the aim of the present study to find out if hygienically relevant bacteria can integrate and persist in ochreous incrustations, and if the water phase, in contact with the ochre, can become contaminated by those bacteria. Furthermore, the effectiveness of disinfection, using hydrogen peroxide, against hygienically relevant bacteria attached to ochre was studied.

Escherichia coli, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Legionella pneumophila* and *Aeromonas hydrophila* were selected as examples of hygienically relevant bacterial species. The persistence of those bacteria was investigated, on the one hand, in suspensions of ochre from dewatering and drinking water wells and in the corresponding well water in microcosm experiments and, on the other hand, attached to ochre under flow-through conditions in column experiments. The disinfection effectiveness of hydrogen peroxide, against *E. coli*, *E. faecalis* or *P. aeruginosa* attached to ochre was tested for H_2O_2 solutions of up to 34 g/l.

Overall, a part of the population of all target bacteria survived in ochre for up to 14 days in a culturable state, both in microcosm and flow-through experiments. In microcosm experiments, *E. coli* showed the most pronounced mean reduction of culturability of about three log units after 14 days, both in ochre suspensions and well waters. In contrast, the concentration of *L. pneumophila* only decreased about one log unit over the course of 14 days. Compared to the survival in ochre suspensions in microcosms, an even larger portion of the populations of the investigated target bacteria survived under flow-through conditions attached to ochre. Considering all target bacteria, the mean log reduction after 14 days was only about 0.5; *P. aeruginosa* showed no reduction at all, whereas the amount of *E. coli* decreased by 1.1 log units. Over the whole period of the experiments (14 days), target bacteria, originating from the contaminated ochre, were detected in the water phase of the ochre containing columns.

The hydrogen peroxide concentration officially recommended for well disinfection, about 150 mg/l, was ineffective against hygienically relevant bacteria (*E. coli*, *E. faecalis* or *P. aeruginosa*) attached to ochre. Even the addition of a single dose of a more than 200 times higher concentrated H_2O_2 solution (up to 34 g/l; contact time: 24 h) resulted only in a minor decline in culturability of about 0.4 to 0.9 log units of the respective bacteria. Only when hydrogen peroxide was added continuously over a time period of 24 h, an appreciable reduction in numbers of culturable target bacteria could be achieved. *E. coli* showed a decrease of 4.4 log units after the continuous addition of hydrogen peroxide solution of 12 g/l; at higher concentrations *E. coli* was no longer detected at all by cultivation. Numbers of culturable *E. faecalis*, however, only declined 3.2 log units at most when H_2O_2 (up to 34 g/l) was added continuously. In the presence of ochre, hydrogen peroxide was rapidly decomposed catalytically. Interestingly, the presence of bacteria (*P. aeruginosa*) also caused a decrease of the H_2O_2 concentration; presumably by the production of high amounts of hydrogen peroxide scavenging enzymes.

In conclusion, ochre can function as a habitat for hygienically relevant bacteria from which the water phase in contact with the ochre is contaminated over a longer period of time. Once a well has been contaminated by hygienically relevant bacteria and ochre incrustations in such a well could not be removed completely during well rehabilitation, an eradication of bacteria of faecal origin and pathogens, attached to ochre, seems not to be achievable just by the addition of a hydrogen peroxide solution (up to 3 % (v/v) application concentration, corresponding to about 34 g/l) into the well.

Zusammenfassung

Grundwässer können durch äußere Kontamination, zum Beispiel in Folge von Starkregen, Überschwemmungen oder raschen Kurzschlüssen mit Oberflächengewässern mit hygienisch relevanten Mikroorganismen und damit auch mit Krankheitserregern verunreinigt werden. Mit dem Grundwasserstrom oder durch direkten Eintrag können solche Mikroorganismen in Brunnen der Trinkwassergewinnung gelangen. Liegt dort eine Inkrustierung vor, meist in Form von Eisen(III)-oxiden bzw. -oxyhydroxiden (Ocker), wird den Organismen eine sehr große und poröse Oberfläche zur Ansiedlung geboten. Ziel der vorliegenden Arbeit war daher zu klären, inwiefern solche Organismen sich in Ocker einnisten und dort persistieren, und somit ein Potential zur Kontamination des Wassers bilden. Des Weiteren wurde die Wirksamkeit einer Desinfektion mit Wasserstoffperoxid gegenüber an Ocker angehefteten hygienisch relevanten Bakterien untersucht.

Als Beispiele hygienisch relevanter Bakterienspezies wurden *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Legionella pneumophila* und *Aeromonas hydrophila* ausgewählt. In Laborversuchen wurde zunächst in Mikrokosmen die Persistenz der Bakterien in Suspensionen von Ocker aus Sümpfungs- und Trinkwasserbrunnen ebenso wie im dazugehörigen Brunnenwasser untersucht. Um die Situation im Brunnen besser abzubilden wurde anschließend das Überleben der Bakterien unter Durchflussbedingungen angeheftet an Ocker in ockerhaltigen Versuchssäulen getestet. Die Desinfektionswirkung von Wasserstoffperoxid wurde gegenüber an Ocker angehefteten *E. coli*, *E. faecalis* oder *P. aeruginosa* mit H_2O_2-Lösungen von bis zu 34 g/l überprüft.

Insgesamt betrachtet überlebte sowohl in Mikrokosmen als auch in Versuchssäulen unter Durchflussbedingungen ein Teil der Populationen der Zielorganismen mindestens 14 Tage in einem kultivierbaren Zustand. In Mikrokosmen zeigte *L. pneumophila* die geringste und *E. coli* die größte durchschnittliche Abnahme der Kultivierbarkeit von etwa einer Log-Stufe bzw. drei Log-Stufen nach 14 Tagen, sowohl in Suspensionen verschiedener Ocker als auch in Brunnenwässern. Unter Durchflussbedingungen, angeheftet an Ocker, überlebte sogar ein noch größerer Teil der Populationen der untersuchten hygienisch relevanten Bakterien, als in Ockersuspensionen im Batch-Ansatz. Die Abnahme der Kultivierbarkeit aller Zielorganismen insgesamt betrachtet betrug hier im Mittel nur ein halbe Log-Stufe nach 14 Tagen, wobei *P. aeruginosa* keine Abnahme, *E. coli* hingegen eine Abnahme von 1,1 Log-Stufen zeigte. In der Wasserphase der ockerhaltigen Versuchssäulen wurden über den gesamten Zeitraum von 14 Tagen die Zielorganismen nachgewiesen, welche dem Ocker entstammten.

Die zur Brunnendesinfektion empfohlene Wasserstoffperoxid-Konzentration, von etwa 150 mg/l, war unwirksam gegenüber an Ocker angehefteten hygienisch relevanten Bakterien (*E. coli*, *E. faecalis* oder *P. aeruginosa*); auch die einmalige Zugabe von mehr als 200-mal höheren Wasserstoffperoxid-Konzentrationen (bis zu 34 g/l; Kontaktzeit: 24 h) führte nur zu einer geringen Abnahme der Kultivierbarkeit der genannten Bakterien um 0,4 bis 0,9 Log-Stufen. Erst bei einer kontinuierlichen Zugabe von H_2O_2 über 24 h konnte eine nennenswerte Bakterien-Reduktion erreicht werden. *E. coli* zeigte bei kontinuierlicher Zugabe von H_2O_2 mit einer Konzentration von 12 g/l eine Abnahme der Kultivierbarkeit um 4,4 Log-Stufen; darüber hinaus war *E. coli* kulturell nicht mehr nachweisbar. Die Menge kultivierbarer *E. faecalis* nahm bei kontinuierlicher Zugabe von H_2O_2 (bis zu 34 g/l) um maximal 3,2 Log-Stufen ab. In der Gegenwart von Ocker zersetzte sich Wasserstoffperoxid rasch und auch die Anwesenheit von Bakterien (*P. aeruginosa*) verursachte eine Abnahme der H_2O_2-Konzentration; vermutlich aufgrund von Produktion großer Mengen H_2O_2-abbauender Enzyme.

Als Fazit ist festzustellen, dass Ocker für hygienisch relevante Bakterien ein Habitat darstellen kann, von dem aus das Wasser, welches mit dem Ocker in Kontakt steht, über einen längeren Zeitraum kontaminiert wird. Findet eine Verunreinigung eines ockerinkrustierten Brunnens durch hygienisch relevante Bakterien statt und liegen auch nach einer Reinigung des Brunnens noch Ockerablagerungen vor, scheint eine Abtötung an Ocker angehefteter, fäkaler Bakterien und Krankheitserreger, nur durch Zugabe von Wasserstoffperoxid-Lösung (bis zu 3 % (v/v) Anwendungskonzentration, entsprechend etwa 34 g/l) in den Brunnen, nicht möglich.

1 Introduction

1.1 Background and scope of the study

Groundwater accounts for 97 % of the global liquid freshwater and is an important source of drinking water in many regions of the world (WHO, 2006). Due to filtration of the water through the soil it contains low amounts of bacteria and nutrients (ULTEE et al. 2004) and is naturally free from pathogenic bacteria and viruses (DOTT et al. 1986). Therefore, groundwater has been considered a safe drinking water resource which requires little or no treatment to be suitable for drinking (KVITSAND & FIKSDAL 2010). However, the validity of this assumption had to be confined due to a number of reports about microbial contamination of groundwater (MACLER & MERKLE 2000, HRUDEY et al. 2003, GALLAY et al. 2006, PEDLEY et al. 2006, SAPKOTA et al. 2007, KVITSAND & FIKSDAL 2010, MCKAY 2011, PAYMENT & LOCAS 2011, HYNDS et al. 2014).

Moreover, an investigation of waterborne disease outbreaks in the US showed that more than half the number of outbreaks was preceded by extreme precipitation events (CURRIERO et al. 2001). This finding is of special concern because climate change is likely to speed up the hydrological cycle and increase the frequency of heavy precipitation events and flooding, which will result in greater contamination of both surface and groundwater (COFFEY et al. 2014, ASHBOLT 2010).

Microbial contamination of water resources though is of special concern as water can act as a vehicle of disease. Classical waterborne pathogens originate in the faeces of humans and animals, and are transmitted by the faecal-oral route of infection. These pathogens are the main concern to public health (PEDLEY et al. 2006), but also other types of microorganisms, which occur naturally in aquatic and soil environments, so called opportunistic pathogens, can be transmitted by water to susceptible hosts.

Contaminated groundwater reaching drinking water wells or direct ingress of hygienically relevant microorganisms can cause the occurrence of these organisms in wells. Intruded pathogens, however, come across a large, porous surface if those wells are affected by iron oxide incrustations (i.e. ochre). As it is known that "bacteria in natural aquatic populations have a marked tendency to interact with surfaces" (COSTERTON et al. 1987), and that on all surfaces in contact with non-sterile water biofilms develop, which can serve as a reservoir for pathogenic microorganisms (WINGENDER & FLEMMING 2011), subject of this study was the investigation if incrustations in wells can act as a habitat for hygienically relevant bacteria.

However, it is known as well that iron can present a hazard to organisms because it can convert less reactive hydrogen peroxide, formed by endogenous metabolism or available exogenously, into more reactive oxygen species by the Fenton reaction (ZEPP et al. 1992, HENLE & LINN 1997, TOUATI 2000, CORNELIS et al. 2011). Therefore, it might also be possible that hygienically relevant bacteria are damaged and inactivated due to oxidative stress triggered by iron, when they come into contact with ochre.

Thus, it was studied if hygienically relevant bacteria can integrate, survive and persist in ochre, and if a contamination of the water phase by those bacteria from out of the ochre takes place. Furthermore, recommenced disinfection with hydrogen peroxide was tested for its effectiveness against hygienically relevant bacteria attached to ochre.

1.2 Iron oxide incrustations in wells

Wells abstracting anaerobic groundwater with high amounts of reduced iron and manganese species are prone to the formation of incrustations in or even around the well, as well as in the pump and rising pipe. Those precipitates result from the oxidation of the water-soluble reduced forms of the metals, yielding water-insoluble products. The redox reactions taking place can proceed abiotically, when the anoxic groundwater gets in contact with oxygen, as well as be mediated by microorganisms (e.g. iron-depositing bacteria, Figure 1).

In fully oxygenated water, at circum-neutral pH, the abiotic oxidation of Fe(II) is rapid, with a half-life of < 1 min; whereas under microaerobic conditions the half-life of Fe(II) can be up to 300-fold longer (EMERSON et al. 2010). Therefore, biotic iron-oxidation is of particular significance in microaerobic environments, and neutrophilic iron oxidizers often colonise the interface between aerobic and anoxic zones in sediments and ground waters (HEDRICH et al. 2011).

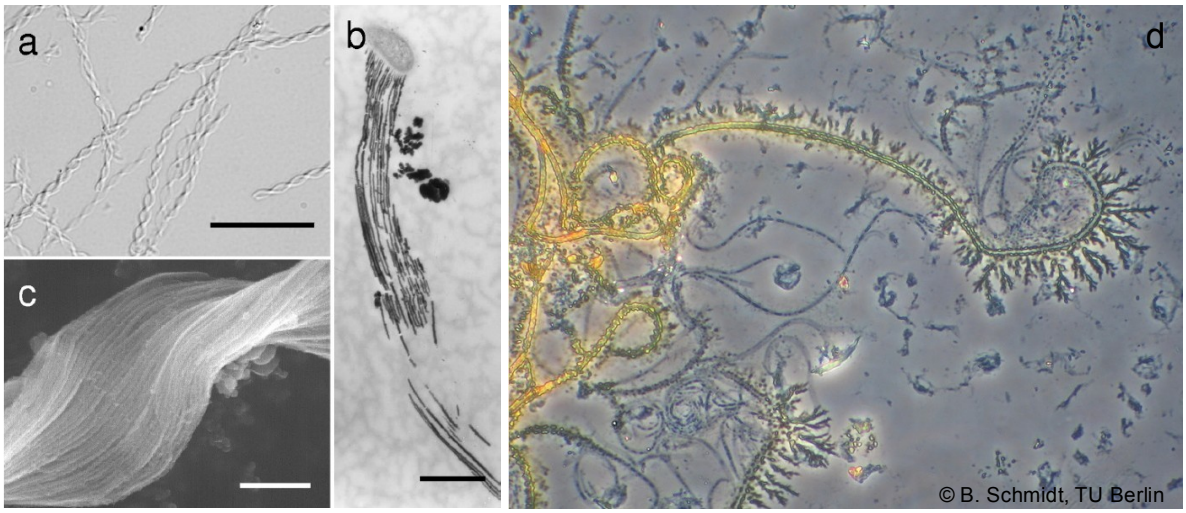

Figure 1: Iron-depositing bacteria. Micrographs of *Gallionella ferruginea* (a-c; SUZUKI et al. 2011) and *Leptothrix* bacteria (d; http://imaggeo.egu.eu/view/452/ by B. Schmidt, TU Berlin). (a) Light micrograph of typical twisted stalks of ocherous flocs collected from a groundwater-receiving tank. Scale bar, 20 µm. (b) TEM image of long stalk fibers originating from the concave side of the bacterial cell. Scale bar, 1 µm. (c) SEM image of closely arrayed, parallel long fibers of the twisted stalk. Scale bar, 0.5 µm. (d) Light micrograph of *Leptothrix* bacteria, known to be capable of oxidizing both iron(II) and manganese(II) producing ferric hydroxide and manganese oxide.

Furthermore, it is important to note that iron may be associated with organic matter, forming organic-metallic complexes, and that in natural environments the purely chemical oxidation process from Fe(II) to Fe(III) is relatively slow (DE MENDONCA et al. 2003). Microbial activity therefore plays a major role in ochre formation in natural environments, since microorganisms, which catalyse the iron precipitation, cause an enormous increase in the reaction speed of the process (DE MENDONCA et al. 2003). This is also true for the precipitation of manganese by manganese-oxidising bacteria, because abiotic oxidation of Mn by dissolved oxygen is very slow at pH < 9.6 (PACINI et al. 2005).

The contribution of biotic iron and manganese oxidation and precipitation in the formation of ochre in wells, as well as in the removal of the metals from raw water during drinking water purification, becomes particularly apparent when bacterial growth is inhibited by bactericidal treatment. This was, for instance, the case with the application of gamma radiation to combat ochre deposition in wells in the former German Democratic Republic. Radiation damage to iron bacteria, in this regard, could be documented microscopically (Figure 2, WISSEL & GERSTNER 1974) and the introduction of bar-shaped radiation sources of Co-60 in the wells (Figure 3) resulted in a stable protective effect against ochre formation (WISSEL et al. 1985).

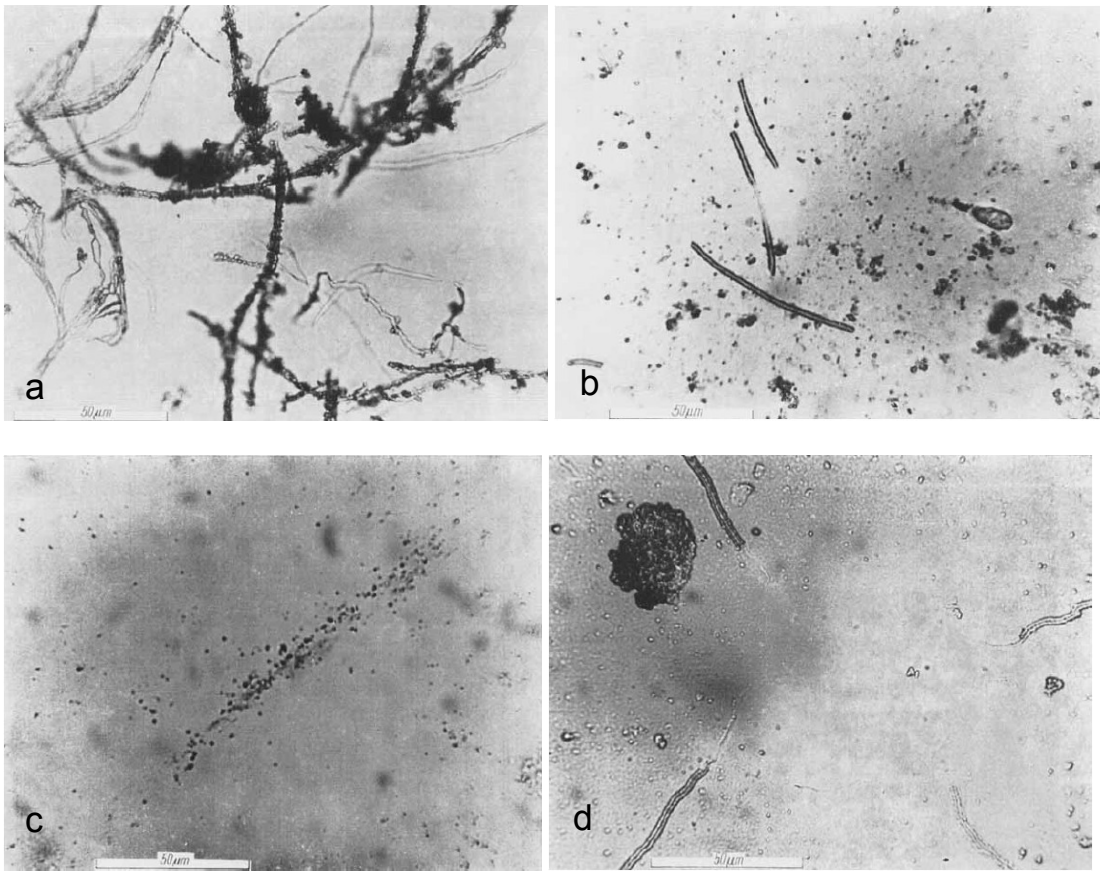

Figure 2: Radiation damage to iron bacteria. Micrographs of iron bacteria (*Leptothrix* spp. and *Gallionella* sp.) on glass slides, (a) after 8 weeks of exposition in a non-irradiated well, (b)-(d) after exposition in an irradiated well (WISSEL & GERSTNER 1974).

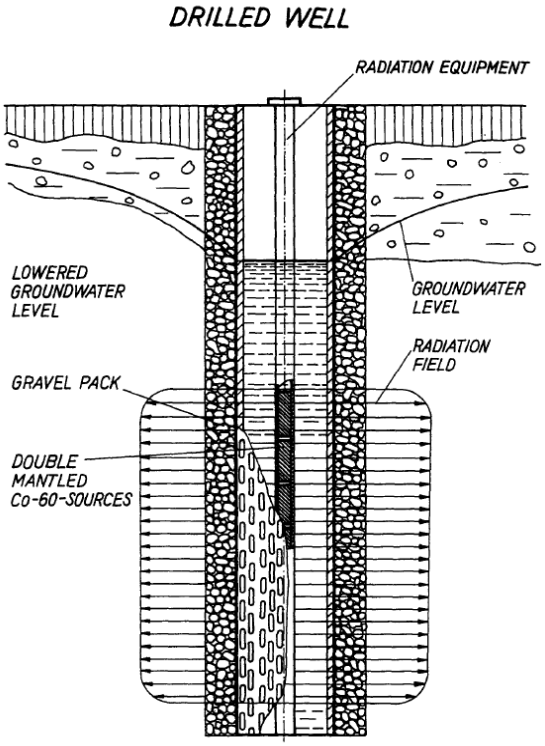

Figure 3: The scheme of a radiation protected well. Application of double Cr-Ni-steel capsuled Co-60-radiation source to kill ochre producing microorganisms; radiation field covering the well filter slots and the adjacent aquifer region (WISSEL et al. 1985).

Furthermore, treatment of trickling filters, to remove iron from potable water, with commercial bleach (NaOCl 5 %), revealed that "the existence of bacteria in the filter can dramatically improve the filter efficiency under the same operating conditions" and that "biological oxidation allows treatment of almost double the iron concentration than physico-chemical oxidation alone" (MICHALAKOS et al. 1997).

SØGAARD et al. (2000) also found the biotically precipitated iron, in drinking-water treatment, being oxidised and precipitated with a rate about 60 times faster than the traditional abiotic process, even in spite of the much poorer physicochemical conditions for the process. Biological phenomena, hence, cause a substantial improvement of the treatment process for the removal of iron and manganese from raw water (MOUCHET 1992).

On the other hand, the formation of precipitates in extraction wells cause a reduced efficiency of the wells, and results in the necessity of expensive and time consuming well regeneration measures (SZEWZYK et al. 2011). Iron-related biofouling is one of the most common problems in water well maintenance (TUHELA et al. 1993). The main morphotypes of iron-depositing bacteria found in groundwater wells are *Gallionella*, *Leptothrix* and *Siderocapsa*, but the taxonomic and phylogenetic assignment of most morphologically described iron bacteria remains unsolved until today (SZEWZYK et al. 2011). Recent investigations revealed a large diversity of iron-depositing bacteria both in natural habitats and in drinking water systems, and besides known ones, such as *Leptothrix*, *Pedomicrobium*, *Pseudomonas* and *Hyphomicrobium*, many isolates were affiliated to taxa for which iron deposition has not been described so far (SZEWZYK et al. 2011).

A survey on well aging performed by the DVGW (German Technical and Scientific Association on Gas and Water) amongst its members revealed that 87 % of the well aging processes were due to incrustations, with a significant dominance of ochreous incrustations (84 % of all incrustations; HOUBEN 2003a). Oxides of Fe(III) represent the most common incrustation type in Germany, and they are characterised by having a large surface area and a high sorption capacity (HOUBEN 2003a).

Figure 4: Ochreous deposits in a drinking water well (left), and on a pump from a dewatering well (right).

In the case of iron oxide formation, the first precipitate is usually ferrihydrite, a phase of low crystallographic order and high surface area (150 – 400 m^2/g), which over time transforms to the thermodynamically more stable phase goethite (α-FeOOH, 20 – 80 m^2/g) (TUHELA et al. 1997, HOUBEN 2003a). The reaction has a half-life of 110 – 160 days in the normal pH-range of groundwater (pH 6-7; HOUBEN 2003a). But the process can be significantly retarded in the presence of inhibitors of crystallisation, such as dissolved silicate or dissolved organic carbon (TUHELA et al. 1997), small amounts of adsorbed phosphate or elevated concentrations of divalent metals, such as Co, Ni, Cu, Zn, in the crystal structure (HOUBEN 2003a). In the field, the transformation process may therefore take several years (HOUBEN 2003a).

Besides, sometimes minor amounts of lepidocrocite (γ-FeOOH, 70 – 80 m^2/g) can be found, but usually only in springs and wells in soils that are relatively poor in silicates (TUHELA et al. 1997, HOUBEN 2003a).

Manganese oxides, in contrast, are much less abundant in ochreous incrustations in wells in Germany than iron oxides (15 % vs. 78 %), and both are rarely found together in one well due to the different redox potentials required for formation (Fe: ca. 0.0 – 0.5 V; Mn: ca. 0.6 – 1.2 V; HOUBEN 2003a). Mn-oxides relevant to groundwater or ambient surface conditions are primarily birnessite and todorokite (TUHELA et al. 1997), which are known to be meta-stable and undergo a crystallisation sequence similar to that of Fe oxides involving de-hydroxylation and a decrease of surface area and reactivity (HOUBEN 2003a).

In biofouling situations in wells, the accumulation of iron- or manganese precipitates, sorbed ions, and organic matter, in form of cells and debris, leads to the development of complex microbial biofilm communities (TUHELA et al. 1997). Those biofilms may act as a reservoir for pathogenic microorganisms, as described for environmental biofilms and biofilms in man-made water systems (KEEVIL 2002, BALZER et al. 2010, WINGENDER & FLEMMING 2011).

1.3 Obligate pathogens in groundwater

1.3.1 Sources of faecal contamination of groundwater

Pathogens of faecal origin can enter groundwater bodies if the aquifer is insufficiently protected from the surface environment and if a source of faecal contamination is present. Sources of faecal contamination can be, depending on the surroundings, leakages from septic tanks, sewers, cesspools or tanks to store manure (Figure 5). These point sources contain faeces in very high concentrations, and may therefore also contain high amounts of pathogens,

but a release of material is associated with a failure of the particular system. Other sources are combined sewer overflows or surface run-off (especially from agricultural land fertilised with manure) during heavy rainstorms and also secondary effluents from waste water treatment plants (Figure 5), which still contain high levels of pathogens and indicator organisms (Table 1).

Table 1: Typical concentrations of enteric pathogens and indicator organisms in raw and treated domestic wastewater (MEDEMA et al. 2003b). [a]Numbers based on cultivation methods may underestimate the numbers of pathogens or indicator organisms actually present due to cultivation bias or because they are present in a non-culturable state.

Microorganism	Raw sewage (numbers[a]/litre)	Secondary effluent (numbers[a]/litre)
Pathogens		
Parasites		
Cryptosporidium sp.	1 000 – 10 000	10 – 1 000
Giardia sp.	5 000 – 50 000	50 - 500
Viruses		
Enteroviruses	10 – 100	1 – 10
Norwalk like viruses	10 – 1 000	1 – 100
Rotavirus	10 – 100	1 – 10
Bacteria		
Salmonella spp.	100 – 10 000	10 – 10 000
Index parameters		
Coliforms	$10^7 – 10^9$	$10^6 – 10^8$
Thermotolerant coliforms / *E. coli*	$10^6 – 10^8$	$10^5 – 10^7$
Enterococci	$10^6 – 10^7$	$10^4 – 10^6$
Clostridium perfringens	$10^5 – 10^6$	$10^4 – 10^5$
F-RNA phages	$10^6 – 10^7$	$10^5 – 10^6$
Somatic coliphages	$10^6 – 10^7$	$10^5 – 10^6$
Bacteroides phages	$10^4 – 10^5$	$10^3 – 10^4$

These sources produce a relatively diffuse pollution, but are, because of sanitation regulations on point sources, the primary source of faecal loading into water courses in developed countries (COFFEY et al. 2014). Furthermore grazing livestock or wild animals can be sources of faecal contamination. Figure 5 illustrates sources of faecal contamination of surface and groundwater and pathways leading to ingress of contaminated water into wells.

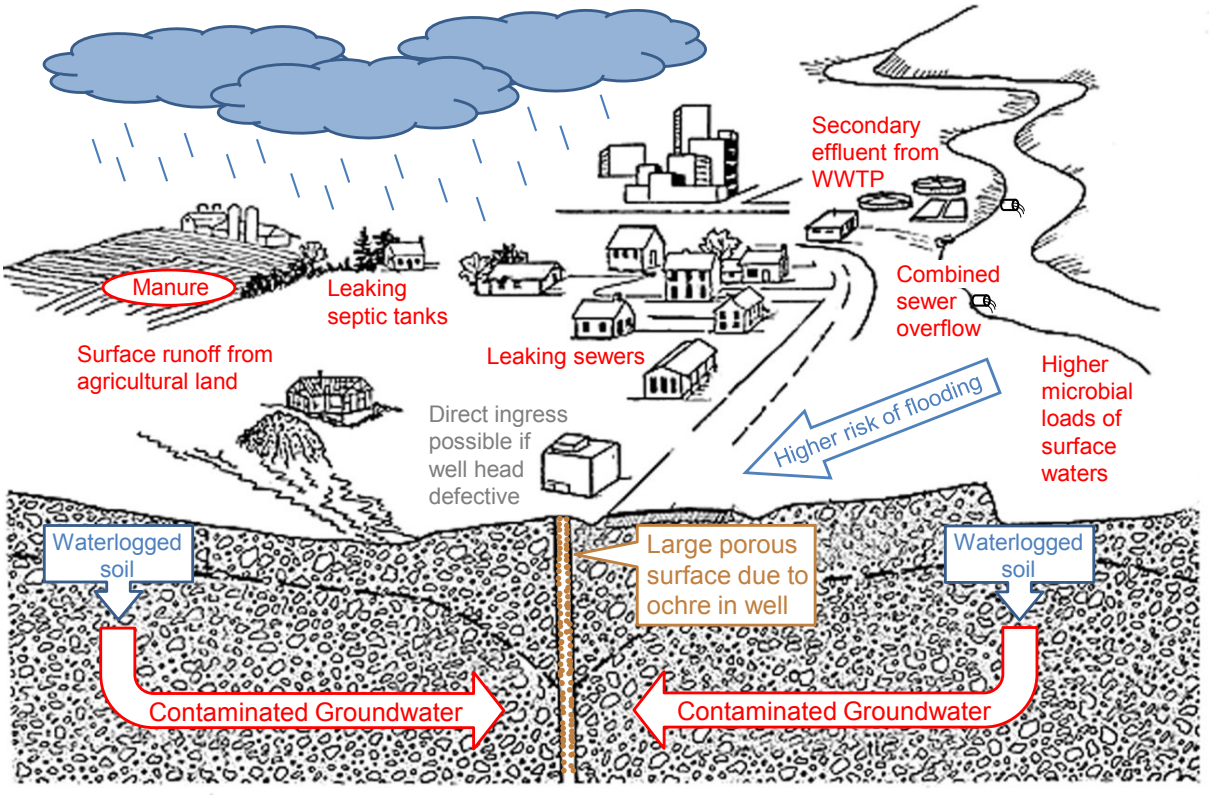

Figure 5: Sources and pathways of faecal contamination of groundwater wells. Extreme precipitation increases the risk of pathogen entry into aquifers due to higher microbial loads in surface waters and faster transport of microorganisms through soil, or by ingress at the wellhead. During prolonged heavy rain the soil becomes waterlogged and provides a hydraulic pathway for the rapid transport of pathogens, and also absorbed organisms can be mobilised (see section 1.4). WWTP: waste water treatment plant. Figure modified after Navajo Nation Environmental Protection Agency, http://navajopublicwater.org/SWAP2.html.

In general, human faecal wastes give rise to the highest risk of waterborne disease, because the probability of human pathogens being present is highest (MEDEMA et al. 2003b). Especially human enteric viruses (such as Norwalk-like caliciviruses, hepatitis A and E viruses, rotavirus and enteroviruses) and *Shigella* spp. in water originate predominantly from human faecal material (MEDEMA et al. 2003b). Whereas other pathogens, such as *Campylobacter* spp., *Salmonella* spp. and *Cryptosporidium* spp., are present in both human and animal wastes (MEDEMA et al. 2003b). Percentages of animals shedding selected zoonotic pathogens are listed in Table 2.

Table 2: Percentage of animals shedding selected zoonotic pathogens (MEDEMA et al. 2003b).

	% of animals shedding pathogens						
Pathogens	**Cattle**	**Calves**	**Sheep**	**Pig**	**Poultry**	**Rodents**	**Waterfowl**
Crytosporidium sp.		20 – 90	8 – 40	5 – 20	9	30	13 – 100
Giardia sp.		57 – 97				10 - 95	6 – 50
Camplylobacter spp.						1 – 10	1 – 10
Salmonella spp.	13		4 – 15	7 – 22			
Yersinia sp.				1 – 10			
Pathogenic *E. coli*	3.5		2	1.5 – 9			

Whether faecally derived pathogens in surface water or soil can reach the groundwater or not mainly depends on the soil composition, the aquifer type, climatic conditions and the type of pathogen present. In general, the faster the recharge of the aquifer and the smaller and more persistent a pathogen, the higher is the probability of its entry into the groundwater (cf. section 1.3.2).

Pollution of a groundwater well can occur by inflow of contaminated groundwater, but also through the well itself. Direct ingress of contaminants into wells can happen due to design defects, such as inadequate sanitary completion of wells and boreholes (PEDLEY et al. 2006), and an insufficient protection of the wellhead zone.

1.3.2 Microbial contamination of groundwater and waterborne disease outbreaks due to contaminated groundwater

Groundwater is often shielded from the immediate influence of microbial contamination by the overlying soil and unsaturated zones (PEDLEY et al. 2006). Therefore, it has traditionally been regarded as a relatively safe source of drinking water because of the natural tendency for pathogens to be removed by straining through small pores, attachment to mineral surfaces or inactivation/die-off during transport (MCKAY 2011). However, the degree of pathogen removal in soils and aquifers can vary greatly due to a number of factors such as material type, depth to the water table, flow rate, faecal concentration in recharge water or types of pathogens present (MCKAY 2011).

Fine-textured soils (clay, silt) retain pathogens better than light-textured ones (sand) and the extended time of travel from the surface to the groundwater in low permeability aquifers promotes pathogen die-off (MEDEMA et al. 2003b). In contrast, microorganisms move more easily through fractured granitic or crystalline rock aquifers, and so karst aquifers with limited

unconsolidated soil overlayers are especially vulnerable to microbial contamination (MAHLER et al. 2000, MACLER & MERKLE 2000, HYNDS et al. 2014). Furthermore, shallower groundwater sources are more readily contaminated than deeper ones.

The smaller size of viruses relative to the larger bacteria and protozoa may allow freer movement through the soil and aquifer. Consequently, in terms of public health, pathogenic viruses from faecal contamination are of most concern for groundwater (MACLER & MERKLE 2000). However, if the filtration capacity of the soil is limited and allows rapid recharge of the aquifer, or if surface water and groundwater are even in direct connection, size exclusion of bacteria and protozoa is no longer given. Because protozoan pathogens, such as *Giardia* and *Cryptosporidium* spp., are shed in faeces as infective thick-walled cysts or oocysts, which remain viable for weeks to months in fresh water and are extremely resistant to processes generally used for disinfection of water, the control of waterborne transmission presents real challenges (WHO 2008).

Several studies have demonstrated the occurrence of faecal indicators and enteric pathogens in groundwater sources (PEDLEY et al. 2006, HYNDS et al. 2014). In the US up to half of the drinking water wells tested had evidence of faecal contamination (MACLER & MERKLE 2000). An investigation of the groundwater contamination in Canada and the US, recorded from 1999 to 2013, by HYNDS et al. (2014) showed that approximately 15 % of the groundwater samples were positive for enteric pathogens. Viruses accounted for 58 %, bacteria for 23 % and protozoa for 20 % of the 102 enteric pathogen records from 55 studies. Enteric pathogen record numbers were as follows:

- **Viruses**, Enterovirus (14), Norovirus (12), Hepatitis A virus (8), Rotavirus (7), Adenovirus (6), Human Enteric Viruses (5), Total CulturableViruses (4), Reovirus (2), Small Round Structured Viruses (1);
- **Bacteria**, Vero-toxigenic *Escherichia coli* O157 (5), *Salmonella* spp. (5), Enteropathogenic *Escherichia coli* non O157 (4), *Campylobacter jejuni* (4), *Arcobacter* spp. (1), *Shigella* spp. (1), *Helicobacter* spp. (1), *Leptospira* spp. (1), *Yersinia* spp. (1)
- **Protozoa**, *Cryptosporidium* spp. (9), *Giardia* spp. (10), *Naegleria fowleri* (1)

Three potential contamination pathways were identified in this study (HYNDS et al. 2014):

1. Precipitation patterns (leading to contamination ingress at the wellhead or rapid subsurface infiltration)

2. Geological pathways (increased contaminant transmissivity via unconsolidated/ fractured aquifer materials or increased runoff coefficients)
3. Inadequate source design/construction (e.g. uncovered wellhead, cracked jointing, absence of sanitary seal, etc.)

Illnesses that may result from infection with pathogens in groundwater, and their severity, vary with the organisms and also the susceptibility of the host. However, the predominant recognised illness is acute gastrointestinal illness (i.e. fever, nausea, diarrhoea, and/or vomiting), which is mostly self-resolving in otherwise healthy people, but may be chronic, severe, or fatal to some people including the elderly, infants, pregnant women, and especially the immuno-suppressed and immuno-compromised (MACLER & MERKLE 2000).

As many groundwater sources are used for public supply with a minimum level of treatment, normally chlorination, or with no treatment at all (PEDLEY et al. 2006), the presence of pathogens in groundwater pose a significant threat to public health. In Norway, for example, where most individual and smaller community waterworks supply groundwater directly from a spring or well without treatment, 43 % of the waterborne outbreaks reported during the period of 1984 – 2007 occurred in groundwater systems (KVITSAND & FIKSDAL 2010). In Sweden, Finland and the US, groundwater accounts for more than half of the reported waterborne outbreaks (KVITSAND & FIKSDAL 2010).

A large outbreak of *E. coli* O157:H7, which was linked to contaminated well water, for instance, happened at a fairground in the state of New York, 1999 (CURRIERO et al. 2001). Likewise, the fatal waterborne disease epidemic in Walkerton, Ontario, in May 2000, in which *E. coli* 0157:H7 and *Campylobacter jejuni* were the pathogens identified as being primarily responsible, was due to the contamination of a shallow (5-8 m) well (HRUDEY et al. 2003). Another large multi-pathogen waterborne outbreak, including *Campylobacter coli*, group A rotavirus and norovirus, which was caused by faecal contamination of a groundwater source and a failure in the chlorination system, occurred in Gourdon, France, in August 2000 (GALLAY et al. 2006).

In the first case, 921 persons reported diarrhoea after attending the Washington County Fair, 65 persons were hospitalised, 11 children developed haemolytic uremic syndrome (HUS; a serious and potentially fatal kidney ailment) and two persons died (ANONYMOUS 1999). In the second example, more than 2,300 individuals experienced gastroenteritis, 65 were hospitallised, 27 developed HUS and seven died.

In the latest example, the number of affected people was estimated to be between 2,400 and 2,900. Those numbers clearly demonstrate that microbial contamination of groundwater pose a significant threat to public health.

1.4 Climatic conditions and microbial groundwater pollution

Besides the hydrogeological setting, climatic conditions play a major role for transport of microbial contaminants to groundwater (MACLER & MERKLE 2000). Especially extreme precipitation and/or snowmelt increase the risk of pathogen entry into aquifers due to higher microbial loads in surface waters and faster transport of microorganisms through soil, or by ingress at the wellhead. During prolonged heavy rain the soil becomes waterlogged and provides a hydraulic pathway for the rapid transport of pathogens, and also absorbed organisms can be mobilised (PEDLEY et al. 2006). In addition, flooding could lead to inundation of infrastructure, including drinking water and sewage treatment facilities (COFFEY et al. 2014).

Higher microbial loads of surface waters during extreme rainfall originate from non-point source runoff, combined sewage overflows, reduced treatment of discharged wastewater, as well as erosion and resuspension of river bottom and drain sediment (KISTEMANN et al. 2002). Bacteria of faecal origin were detected in river biofilms (epilithic biofilms, sediments) in approximately two orders of magnitude higher concentrations compared to the overlying water, and the occurrence of opportunistic pathogens was proven (BALZER et al. 2010). Furthermore, not only the microbial load of surface waters is elevated during rainstorms, but also the 'self-purification' of water (e.g. by sedimentation, sunlight inactivation, predation and starvation) becomes much less significant under rapid flow conditions (MEDEMA et al. 2003b).

CURRIERO et al. (2001) investigated the association between extreme precipitation and waterborne disease outbreaks in the US and found out that for the 548 reported outbreaks from 1948 through 1994

- 51 % were preceded by precipitation events above the 90th percentile, and 68 % above the 80th percentile
- outbreaks due to surface water contamination showed the strongest association with extreme precipitation during the month of the outbreak
- a 2-month lag applied to groundwater contamination events

Of the 548 waterborne disease outbreaks about 36 % were known to be from groundwater contamination and about 24 % from surface water contamination; the other 40 % had an unknown water contamination source.

The findings that a good portion of waterborne outbreaks is associated with extreme precipitation is of special concern because climate change (i.e. a rise in air temperatures) is likely to speed up the hydrological cycle and increase the frequency of heavy precipitation events and flooding, which will result in greater contamination of both surface and groundwater (COFFEY et al. 2014, ASHBOLT 2010). Besides this, higher air and water temperatures may lead to the introduction of new pathogens, vectors, and/or intermediary hosts (COFFEY et al. 2014). Those combined changes in rainfall and temperature, together with changes in human land use and agricultural practices, will have important impacts on faecal bacteria prevalence, dispersion, and exposure routes (COFFEY et al. 2014).

Effects of future precipitation, temperature, agricultural production systems and land use on microbial fate and transport to water sources are summarised in Figure 6 (COFFEY et al. 2014).

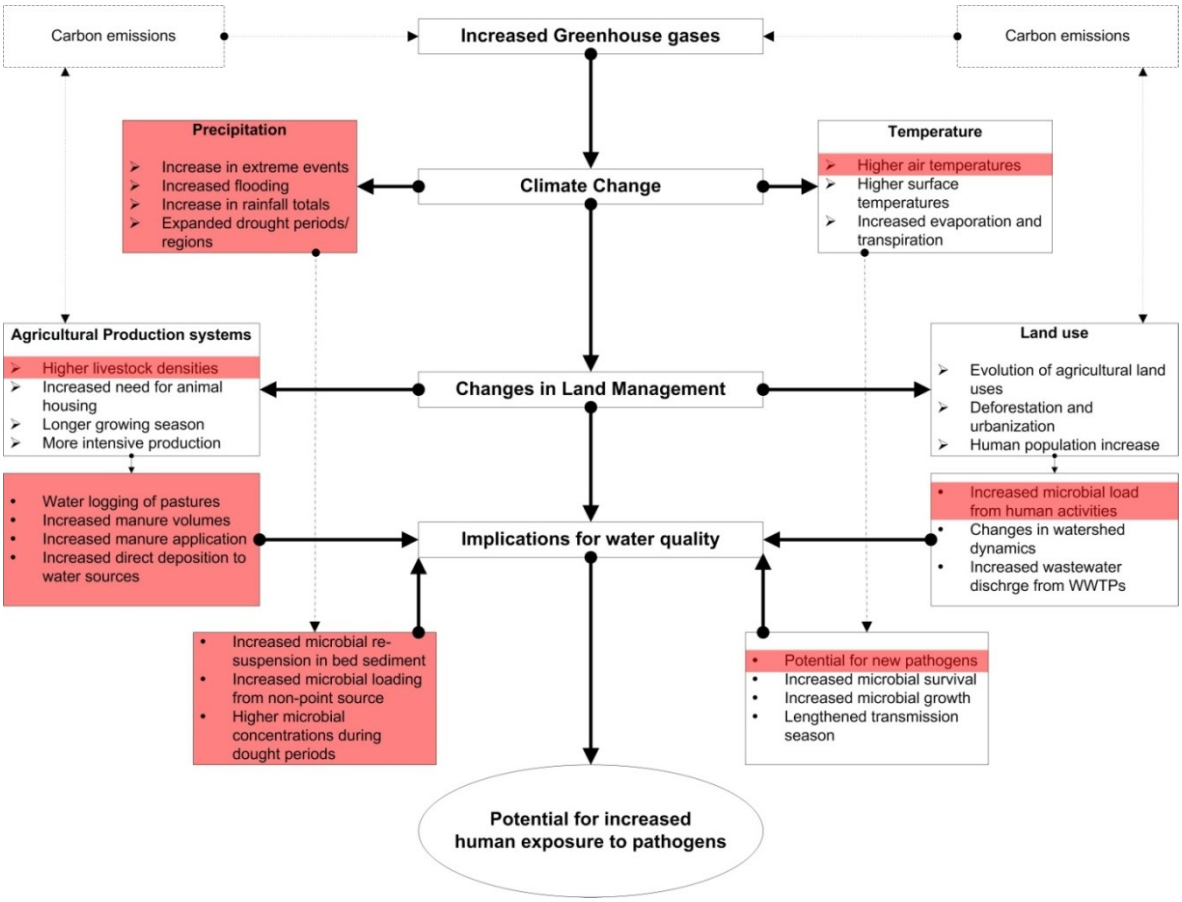

Figure 6: Consequences and impacts of key climate change factors on microbial water quality (according to COFFEY et al. 2014). Highlighted are factors of particular relevance for an increased occurrence of pathogens in groundwater.

1.5 Hygienically relevant microorganisms

1.5.1 Waterborne pathogens and indicator organisms

Two categories of hygienically relevant microorganisms in drinking water systems can be distinguished, (1.) microorganisms with pathogenic properties and (2.) indicator organisms (WINGENDER & FLEMMING 2011).

Waterborne pathogens comprise of pathogenic bacteria, obligate (e.g. *Shigella* spp.) and opportunistic ones (e.g. *Legionella* spp.), viruses (e.g. Human caliciviruses) and parasites (e.g. *Cryptosporidium parvum*). Obligate waterborne pathogens are introduced into the environment by the faeces of infected hosts (humans and animals), in which they mostly initiate infection of the gastrointestinal tract following ingestion; these pathogens do not grow in water (WHO 2008). However, opportunistic pathogens are environmental organisms which can grow in water and soil (WINGENDER 2011). Major routes of transmission in those cases are inhalation of or contact with contaminated water, leading to infections of the respiratory tract or exposed sites (e.g. wounds, mucous membranes, eyes) (WHO 2008). An overview of waterborne pathogens, their sources and persistence in water, as well as symptoms of illness associated with those pathogens and infective doses are given in Table 3.

Table 3: Waterborne pathogens. Adapted from WHO 2008 and ASHBOLT 2010.

Group of Organism	Source(s)	Symptoms	Persistence in water[(a)]	Infective doses[(b)]
Viruses				
Adenovirus	human faeces	C Co F G H R	> 1 month	$1 – 10^2$
Astrovirus	human faeces	G	> 1 month	$1 – 10^2$
Norovirus	human faeces	G	> 1 month	$1 – 10^2$
Coxsackie A&B	human faeces	C E-M F H R S	> 1 month	$1 – 10^2$
Echovirus	human faeces	C E-M F G R P S	> 1 month	$1 – 10^2$
Hepatitis A virus	human faeces	H	> 1 month	$1 – 10^2$
Hepatitis E virus	pig/human faeces[(c)]	H A	> 1 month	$1 – 10^2$
Poliovirus	human faeces	C F E-M P R	> 1 month	$1 – 10^2$
Rotavirus	an./human faeces[(c)]	G	> 1 month	$1 – 10^2$
Bacteria				
Aeromonas spp.	water, soil, food, sewage, faeces	G? W R, septicaemia	"indigenous"	$> 10^4$

Campylobacter jejuni	an./human faeces	G-F	1 week to 1 month	$10^2 – 10^4$
enterohaemorrhagic *E. coli* (EHEC)	an./human faeces	G, kidney failure	1 week to 1 month	$1 – 10^2$
Legionella spp.	freshwater, biofilms/amoebae	R	"indigenous"	$10^2 – 10^4$
Mycobacterium avium complex	freshwater/biofilms	R, weight loss	"indigenous"	$> 10^4$
Mycobacterium marinum	sea water	S W, granuloma	"indigenous"	$> 10^4$
Pseudomonas aeruginosa	water, soil, sewage, faeces	E&E R S U W, septicaemia	"indigenous"	$> 10^4$
Salmonella spp.	an./human faeces	G-F	1 week to 1 month	$> 10^4$
Shigella spp.	an./human faeces	Bloody diarrhoea	up to 1 week	$1 – 10^2$
Vibrio spp.	seawater, faeces, aquatic organisms	G W	up to 1 week to over a month[(d)]	$> 10^4$
Yersinia enterocolitica	an./human faeces	Appendicitis-like G	> 1 month	$> 10^4$
Protozoa				
Acanthamoeba spp.	Aquatic environments, soil, biofilms	keratitis, uveitis	"indigenous"	$1 – 10^2$
Cryptosporidium parvum	an./human faeces	Watery diarrhoea F	> 1 month	$1 – 10^2$
Entamoeba histolytica	human faeces	G/dysentery	1 week to 1 month	$1 – 10^2$
Giardia intestinalis	an./human faeces	G/bloating	1 week to 1 month	$1 – 10^2$

[(a)]Detection period for infective stage in water at 20 °C

[(b)]Relative infectivity: From experiments with human volunteers, from epidemiological evidence and from animal studies. Infective doses in numbers of organisms or particles.

[(c)]Enteric viruses from humans cause most waterborne viral infections (i.e. animal viruses from the same group/family do not infect humans and vice versa, with possible exceptions of porcine hepatitis E and bovine Rotavirus & Norovirus)

An., animal source, largely mammals/birds that may yield human-infectious strains; A, abortion; C, carditis; Co, conjunctivitis; E&E, infections of ears and eyes; E-M, encephalitis-meningitis; F, fever; G, gastroenteritis; G-F, gastro+fever; H, hepatitis; P, paralysis; R, respiratory infection; S, skin infection; U, urinary tract infection; W, wound infection.

Owing to the multitude of different waterborne pathogens it is practically and financially not feasible to test for all pathogens in routine evaluation of the microbiological safety of drinking water. Therefore, different indicator organisms are utilised in microbial water quality testing.

The German Drinking Water Ordinance, for instances, defines limit values for *E. coli* and enterococci (both 0/100 ml) and comprises as microbial indicator parameters *Clostridium perfringens* (incl. spores), coliform bacteria and colony counts 22 °C and 36 °C (ANONYMOUS 2001a).

1.5.2 Indicators of faecal pollution

There are on the one hand true indicators of faecal pollution, like *Escherichia coli* or intestinal enterococci, which detection indicates the probability of the presence of faecally derived pathogens and therefore a potential health risk (PAYMENT & LOCAS 2011). Criteria for a microbial faecal indicator have been defined as follows (MEDEMA et al. 2003a):

- Should be absent in unpolluted water and present when the source of pathogenic microorganisms of concern is present
- Should not multiply in the environment
- Should be present in greater numbers than the pathogenic microorganisms
- Should respond to natural environmental conditions and water treatment processes in a manner similar to the pathogens of concern
- Should be easy to isolate, identify, and enumerate
- Should be inexpensive to test thereby permitting numerous samples to be taken
- Should not be a pathogenic microorganism (to minimize the health risk to analysts)

Escherichia coli is present in very high numbers in human and animal faeces, rarely found in the absence of faecal pollution and is considered the most suitable indicator of faecal contamination (WHO 2008). *E. coli* is a Gram-negative, oxidase-negative, catalase-positive straight rod, a member of Enterobacteriaceae and belongs to the thermotolerant coliforms that are able to ferment lactose at 44 – 45 °C (CABRAL 2010, WHO 2008). *E. coli* can be differentiated from the other thermotolerant coliforms by the ability to produce indole from tryptophan or by the production of the enzyme β-glucuronidase (WHO 2008).

Intestinal enterococci, although their numbers in human faeces are about an order of magnitude lower than those of *E. coli*, have the advantage that they tend to survive longer in water environments than *E. coli* (WHO 2008) and their persistence patters are similar to those of potential waterborne pathogenic bacteria (FIGUERAS & BORREGO 2010). HYNDS et al. (2014) found that both protozoan and viral pathogens were correlated with the presence of intestinal

enterococci. Therefore, routine basic microbial analysis of drinking water, testing for *E. coli* by culture methods, should be complemented with the quantification of enterococci (CABRAL 2010).

Intestinal enterococci comprise the species *Enterococcus faecalis*, *E. faecium*, *E. durans* and *E. hirae*, and are a subgroup of the larger group of organisms defined as faecal streptococci (WHO 2008). Faecal streptococci are Gram-positive, catalase-negative, non-sporeforming cocci that grow in a medium containing bile salts and sodium azide. Intestinal enterococci are faecal streptococci that grow in the presence of 6.5 % NaCl at 45 °C (CABRAL 2010). In human faeces almost exclusively two enterococci species are present, *E. faecalis* and *E. faecium*, which are also the predominant species in sewage, whereas in animal faeces other species co-occur (such as *E. avium*, *E. cecorum*, *E. durans*, *E. gallinarum* and *E. hirae*; CABRAL 2010, FIGUERAS & BORREGO 2010).

1.5.2.1 Pathogenic potential of E. coli and E. faecalis

Even though *E. coli* is a harmless and common inhabitant of the human intestinal tract, it must be noted that it can cause serious disease in other parts of the body (e.g. urinary tract infections or bacteraemia, WHO 2008). Besides, there are some strains of *E. coli* that are obligate pathogens, such as enterohaemorrhagic or enterotoxigenic *E. coli* (EHEC, ETEC), which have been involved in waterborne outbreaks (WINGENDER 2011). For instance, *E. coli* O157:H7, the most prominent representative of the EHEC, which produces Shiga toxin, can cause haemorrhagic colitis with bloody diarrhoea and haemolytic uraemic syndrome, especially in children (SZEWZYK et al. 2000).

Enterococci are commensal bacteria, but can also act as opportunistic pathogens (SAPKOTA et al. 2007) and are among the leading causes of nosocomial infections (SZEMES et al. 2010). Enterococcal infections include urinary tract infections, hepatobiliary sepsis, endocarditis, surgical wound infections, bacteraemia, and neonatal sepsis (AUFFRAY et al. 2011). While *E. faecalis* is the most common species isolated from human illnesses, *E. faecium* seems to be linked with higher threat of antibiotic resistance (AUFFRAY et al. 2011). Among acquired resistances, vancomycin-resistant enterococci (VRE) constitute the most serious concern that has emerged in human clinical infections (AUFFRAY et al. 2011). VRE have been isolated from sewage, waste water treatment plants and rivers, and hospital effluents have been identified as a significant source of ciprofloxacin- and vancomycin-resistant enterococci (VARELA et al. 2013). Furthermore, high levels of erythromycin, tetracycline, and clindamycin re-

sistance was observed in *Enterococcus* spp. recovered from surface water and groundwater situated down gradient from a concentrated swine feeding operation (SAPKOTA et al. 2007).

1.5.3 Indicators to measure the effectiveness of water treatment processes

Other indicator organisms, as the faecal indicators, are general indicators of water quality and treatment efficiency, such as total coliforms and heterotrophic plate counts (HPC).

Total coliform bacteria are facultative anaerobic, Gram-negative, oxidase-negative, non-sporeforming rods that ferment lactose with acid and gas production in 24 to 48 h at 35 – 37 °C in the presence of relatively high concentrations of bile salts (CABRAL 2010, FIGUERAS & BORREGO 2010, WHO 2008). As part of the lactose fermentation, total coliforms produce the enzyme β-galactosidase, and they comprise both faecal and environmental species (WHO 2008). Coliforms should be absent from adequately treated plant effluents and their presence in the distribution system could be due to cross-connections or failure to maintain an adequate disinfectant residual (FIGUERAS & BORREGO 2010).

HPC measurements detect a wide spectrum of heterotrophic microorganisms, including bacteria, yeasts and moulds, based on their ability to grow on non-selective media, over a specified incubation period and at a defined temperature (WHO 2008). HPC measurement, although there is no universal method (differences in media, incubation temperature and periods), can be used to assess the cleanliness and integrity of distribution systems and the presence of biofilms (WHO 2008).

1.5.4 Opportunistic pathogens

Coliform bacteria, however, are not only relevant as indicator parameter, some of which can also act as facultative pathogens in nosocomial infections, especially *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp. and *Serratia* spp. (FEUERPFEIL et al. 2009).

Klebsiella pneumoniae accounts for up to 10 % of all nosocomial bacterial infections, which can occur at nearly any body site, even though urinary and respiratory tract infections predominate (STRUVE & KROGFELT 2004).

Most relevant opportunistic bacterial pathogens linked to water-related diseases though are *Legionella pneumophila* and some other *Legionella* species, *Pseudomonas aeruginosa* and non-tuberculous mycobacteria (WINGENDER & FLEMMING 2011).

Legionella pneumophila is a Gram-negative coccobacillus belonging to the gamma-subgroup of proteobacteria (GOMEZ-VALERO et al. 2009). *Legionell*a species are autochthonous members of many natural aquatic environments where they occur in relatively low numbers. However, they can thrive in certain technical aquatic environments, which provide suitable temperatures and conditions for their multiplication (e.g. cooling towers, evaporative condensers, whirlpools, showerheads; WHO 2008, WINGENDER 2011). Growth of *Legionella* spp. is aided by co-existing microorganisms, which provide nutrients, and by free-living amoeba, in which they can reside and multiply (GOMEZ-VALERO et al. 2009). Inhalation of aerosols containing *Legionella* can cause Legionnaires' disease, a severe pneumonia, or Pontiac fever, a flu-like infection. Although all *Legionella* species are considered potentially pathogenic for humans, the clinically most important species is *Legionella pneumophila* (WHO 2008, WINGENDER 2011).

Pseudomonas aeruginosa is a Gram-negative, catalase- and oxidase-positive rod belonging to the family Pseudomonadaceae. It is a common environmental organism and can be found in soil, water, sewage and faeces. *P. aeruginosa* is able to grow and multiply in a wide range of water sources including river, sea and waste water (PIRNAY et al. 2005). In nosocomial infections it is, besides *Staphylococcus aureus*, the pathogen of outstanding importance causing pneumonia, urinary tract and wound infections of which up to 50 % are water-associated (EXNER et al. 2007). Within hospitals *P. aeruginosa* has been isolated from a multitude of moist environments like sinks, water outlets and baths, flower vases, cleaning equipment and even common antiseptics (PIRNAY et al. 2005).

Other human opportunistic pathogens are the Gram-negative, oxidase-positive, rod-shaped bacteria of the genus *Aeromonas*, which belong to the family Vibrionaceae. *Aeromonas* spp. can be found in a variety of fresh and brackish waters and are therefore "considered to be almost synonymous with water and aquatic environments" (JANDA & ABBOTT 2010). Beside surface waters (rivers, lakes, ponds, estuaries), *Aeromonas* spp. were also isolated from groundwater, drinking water and drinking water distribution systems (JANDA & ABBOTT 2010, PERCIVAL et al. 2004). However, the role of drinking water in the transmission of *Aeromonas* infections is still under debate (PERCIVAL et al. 2004). *Aeromonas* spp. can cause wound and respiratory tract infections as well as septicaemia, especially in immunocompromised patients (WHO 2008). Although the gastrointestinal tract is by far the most common anatomic site from which aeromonads are recovered, their role in bacterial gastroenteritis is still controversial (JANDA & ABBOTT 2010).

1.6 Bacterial growth on surfaces: Biofilms

Attachment of bacteria to surfaces and its stimulating effect on microbial activity, especially in oligotrophic environments containing nutrients in colloidal or poorly soluble form, has already been described several decades ago (ZOBELL 1943). Since then, bacterial growth on surfaces, later on termed biofilms, and the associated beneficial effect for bacteria has extensively been studied demonstrating that on all surfaces in contact with non-sterile water biofilms develop (WINGENDER & FLEMMING 2011). Biofilms develop in soil and aquatic environments, just as on tissues of plants, animals and humans, and also in all types of man-made water systems (COSTERTON et al. 1987). The ecological advantages of the biofilm mode of life can be summarised as follows (FLEMMING 2008):

- Formation of stable microconsortia
- Biodiversity: Gradients create different habitats
- Gene pool and facilitated genetic exchange
- Retention of extracellular enzymes in matrix
- Access to particulate biodegradable matter by colonisation
- Recycling of nutrients because lysed cells are retained in the biofilm
- Protection against biocides and other stress
- High population density: Threshold concentration of signalling molecules easily reached, facilitated intercellular communication

In terms of public health, biofilms are of concern, because in recent years it has become obvious that microorganisms with pathogenic properties can persist and multiply in biofilms of man-made water systems (WINGENDER 2011). Biofilms in water storage tanks, drinking-water distribution systems, and domestic plumbing systems can function as a reservoir for pathogens, in which they maybe even protected against disinfectants, and then present a health risk when pathogens are released from the biofilms and transmitted to a susceptible human host (WINGENDER 2011, WINGENDER & FLEMMING 2011, KEEVIL 2002).

Not only surfaces of tanks and pipes can be colonised by microorganisms, but also soft pipeline deposits, mainly composed of iron, manganese, aluminium and calcium, are key sites for microbial growth in drinking water distribution systems (ZACHEUS et al. 2001). Those deposits may support survival of hygienically relevant bacteria that subsequently appear sporadically in the free water phase (SZEWZYK et al. 2011). Therefore, incrustations and deposits in drinking water wells may also provide micro-habitats for pathogens, where they may persist

and survive, even in the presence of disinfectants, and are then a potential source of perseverative water contamination.

1.7 Well rehabilitation

The formation of incrustations in wells negatively affects the performance of wells by reducing its water permeability (HOUBEN 2003a). Incrustations can be formed at well screens, in pumps and rising pipes, as well as in or even around the gravel pack (annulus). A variety of mechanical, hydromechanical, and chemical techniques can be applied to remove incrustations (HOUBEN 2003b). But deposits outside the annulus are practically out of reach for all current mechanical and probably also chemical rehabilitation techniques (HOUBEN & WEIHE 2010).

An overview of processes for well rehabilitation is given in the tables in the annex in section 6.1. Those procedures are namely cleaning by brushes or pumping-out and well sump cleaning, intensive abstraction, piston, CO_2 injection, low-pressure interior flushing, high-pressure flushing procedures, shock wave- or pulse methods and chemical processes using multiple chamber devices (ANONYMOUS 2007).

1.8 Disinfection of wells

In case the bacteriological examination of the raw water, after a well rehabilitation, yields a result exceeding the limit values of the German Drinking Water Ordinance, a disinfection of the well might be necessary (ANONYMOUS 2007). Normally, hydrogen peroxide should be used with an application concentration of about 150 mg/l (ANONYMOUS 2001b).

In the United States and in Canada, in contrast, chlorination is used for well disinfection. The method of shock chlorination involves the introduction of liquid sodium hypochlorite or powdered calcium hypochlorite, and then allowing this treated water to run through the household water distribution system to eliminate all potential bacterial reservoirs (EYKELBOSH 2013). Disinfection is recommended for all new wells and old wells after maintenance, for wells tested positive for total coliform bacteria and/or *E. coli*, and annually or semi-annually as a preventative treatment (ARTIOLA et al 2013, EYKELBOSH 2013). Free chlorine concentration inside the well should be between 200 and 300 mg/l (ARTIOLA et al 2013) and the recommended disinfection times range from 8-48 h, with a mean minimum disinfection time of 12 h (EYKELBOSH 2013).

The German worksheet (ANONYMOUS 2001b), on the contrary, does not recommend sodium or calcium hypochlorite for well disinfection due to formation of chlorination by-products. Those by-products, mainly halogenated trihalomethanes (THMs) and haloacetic acids (HAAs), are formed because during disinfection chlorine also reacts with the natural organic matter (NOM) present in the water (GOPAL et al. 2007). Disinfection by-products, though, have been found to be associated with adverse health effects, if ingested regularly. They can affect the nervous system and increase the risk of cancer, and have also been associated with reproductive disorders (ARTIOLA et al. 2013, GOPAL et al. 2007). Furthermore, studies have also shown that shock chlorination may temporarily increase the concentration of some metals (such as Pb, Cu, Zn, Fe, As) in well water (ARTIOLA et al. 2013). Therefore, the American guideline for shock chlorination explicitly emphasises the importance to flush residual chlorine and any toxic chemicals that may have been formed or released from the well components or aquifer material. Shock chlorinated wells should be purged at least four well volumes, or until no residual chlorine in the well water is detected (ARTIOLA et al. 2013).

1.8.1 Periodic disinfection in order to retard well ageing

On the other hand, the German work sheet on well rehabilitation (DVGW W 130, ANONYMOUS 2007) recommends a periodic disinfection of wells, e.g. a monthly treatment with hydrogen peroxide with an application concentration of about 150 mg/l, to decelerate the biotic formation of ochreous deposits in wells. For that purpose the well is in most cases shut down for a day, the hydrogen peroxide is introduced into the well through the wellhead and distributed throughout the whole volume of the well.

The Berliner Wasserbetriebe, for instance, apply a preventive well treatment using hydrogen peroxide solutions of 1-2 %, yielding a target concentration of about 300 ppm, if evenly distributed in the whole water column of the well, or locally a much higher concentration of about 10,000 ppm, if only added at one spot (just above the upper edge of the filter; H. Schwarzmüller, KWB, KompetenzZentrum Wasser Berlin, person. comm.). A scheme of the treatment procedure is illustrated in Figure 7.

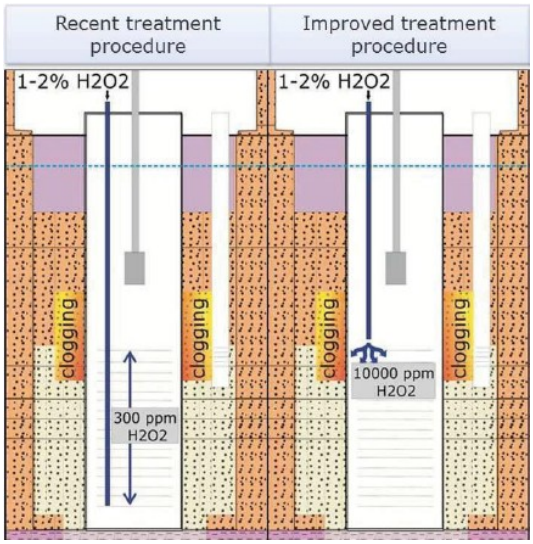

Figure 7: Preventive well treatment using H_2O_2. Addition of H_2O_2 either evenly distributed in the whole water column of the well (left) or locally just above the upper edge of the filter (right; according to SCHWARZMÜLLER et al. 2013).

Dissolution of deposits by hydrogen peroxide, a strong oxidising agent, is based upon its microbicidal effect and the mineralisation of organic material (ANONYMOUS 2007, HOUBEN 2003b). Moreover, when hydrogen peroxide is injected into the well, it will spontaneously decompose exothermically into water and oxygen causing a vigorous and turbulent flow due to the rising gas bubbles, which is thought to have a scrubbing effect on the surfaces inside the well (BRASSINGTON et al. 2009).

Practical experience confirmed structural changes of deposits at pumps of hydrogen peroxide treated wells and within the wells itself (SCHWARZMÜLLER et al. 2013). Besides, in mini column experiments, in H_2O_2-treated columns the deposit formation rate was lower and iron and manganese precipitates could easily be removed by flushing (SCHWARZMÜLLER et al. 2013). However, in a validation phase of the H_2O_2-treatment procedures shown in Figure 7, all three wells, two treated monthly with H_2O_2 following the displayed procedures and one not treated well, showed a loss of specific capacities after a period of one year. But the well treated with H_2O_2 following the improved procedure retained the best specific capacity, whereas the untreated well showed the highest loss of specific capacity (SCHWARZMÜLLER et al. 2013).

1.8.2 Hydrogen peroxide, iron and oxidative stress

The detrimental effect of hydrogen peroxide to bacteria or cells is caused by its generation of oxidative stress. Oxidative stress arises when the concentration of reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the highly re-

active hydroxyl radicals ($^{\bullet}OH$), increases to a level that exceeds the cell's defence capacity (CABISCOL et al. 2000).

Figure 8 illustrates reactions of intracellular ROS, leading either to their formation, which is linked to potential DNA damage, or their decomposition by scavenging enzymes.

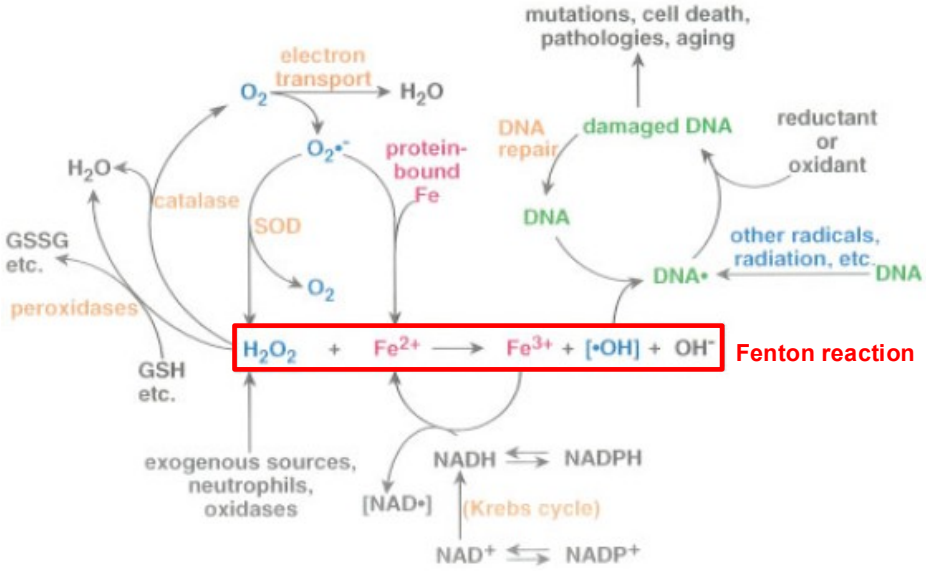

Figure 8: Cellular reactions leading to oxidative damage of DNA via the Fenton reaction. H_2O_2 is formed by endogenous metabolism or is available exogenously. Superoxide, $O_2^{\bullet-}$, is produced as a by-product of O_2 reduction in the electron transport chain. Superoxide dismutation (by superoxide dismutase, SOD) and release of protein-bound iron by superoxide form H_2O_2 and Fe^{2+}, respectively, which in turn can react to form $^{\bullet}OH$-type oxidant(s). These oxidant(s) may cause DNA damage. Fe^{3+} produced by the Fenton reaction is thought to be reduced by available NADH, thus replenishing Fe^{2+}. H_2O_2 can be depleted by catalase or by peroxidases, which utilize reduced glutathione, other thiols, cytochromec, ascorbate, etc. (After HENLE & LINN 1997, modified).

ROS are inevitable by-products of aerobic metabolism. A mixture of superoxide and hydrogen peroxide, i.e. partially reduced forms of oxygen, is formed inside cells when molecular oxygen abstracts electrons from exposed redox moieties of electron-transfer enzymes, in particular from flavoenzymes, which are both ubiquitous and abundant (IMLAY 2008). All aerobic organisms thus experience a steady flux of endogenously generated oxidants (IMLAY 2008). Measurements in *E. coli* for example demonstrated that in well-fed cells H_2O_2 is formed at a constant rate of 10 – 15 µM/s (MISHRA & IMLAY 2012). Therefore, virtually all organisms maintain high titers of enzymes that scavenge superoxide and hydrogen peroxide, especially superoxide dismutase, peroxidases and catalases (IMLAY 2008). Furthermore, most microbes induce additional responses when elevated levels of $O_2^{\bullet-}$ and H_2O_2 are present in their environment (IMLAY 2008).

H_2O_2, since it is an uncharged species, can penetrate membranes with a permeability coefficient similar to that of water (MISHRA & IMLAY 2012). The toxicity of hydrogen peroxide stems from the oxidation and inactivation of solvent-exposed iron-sulphur clusters of dehydratases, as well as of non-redox enzymes that employ a single ferrous iron atom as a substrate-activating factor, thereby blocking key pathways requisite for energy production and biosynthesis (MISHRA & IMLAY 2012). Figure 9 gives an overview of hydrogen peroxide sources and of its detrimental intracellular effects.

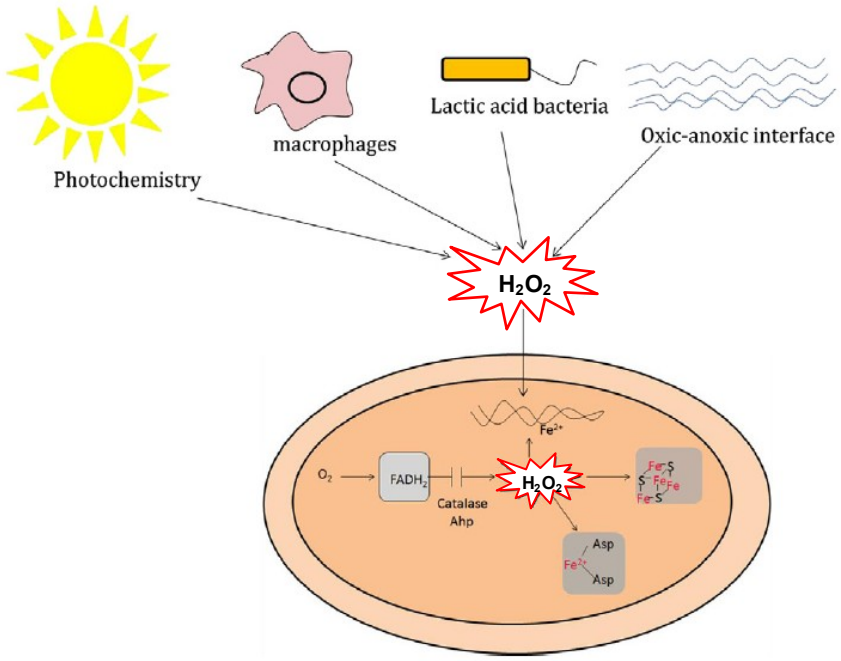

Figure 9: Hydrogen peroxide – sources and detrimental effects to bacteria. Exogenous sources of H_2O_2 include photochemically driven redox reactions, NADPH oxidase responses of plants and macrophages, H_2O_2-excreting microbes such as lactic acid bacteria, and chemical thiol/metal oxidations that occur at oxic-anoxic interfaces. Endogenous H_2O_2 is constantly formed by the adventitious oxidations of flavoenzymes. The H_2O_2 damages DNA through the Fenton reaction, and it also disables dehydratases that contain iron-sulfur clusters and non-redox mononuclear enzymes that contain iron (After MISHRA & IMLAY 2012, modified).

Besides, hydrogen peroxide can react with ferrous (Fe^{2+}) or ferric (Fe^{3+}) iron, which is assumed to lead to the formation of radicals according to the following reactions (SPUHLER et al. 2010, Brassington et al. 2009, DUNFORD 2002):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-} \quad \text{(Fenton-reaction)}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^{\bullet} + H^{+}$$

However, some authors doubt the formation of radicals and instead propose ferryl ion (Fe(IV) species) formation (DUNFORD 2002, HENLE & LINN 1997):

$$Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} + H_2O$$

Or express the possibility that the Fe(IV) species is a short-lived intermediate, which in turn could give rise to $^{\bullet}OH$ (HENLE & LINN 1997, ZEPP et al. 1992):

$$FeO^{2+} + H^{+} \rightarrow FeOH^{3+} \rightarrow Fe^{3+} + {}^{\bullet}OH$$

Hydrogen peroxide may also react with Ferric iron to form $O_2^{\bullet-}$ via the following reaction (HENLE & LINN 1997):

$$Fe^{3+} + H_2O_2 \rightarrow (FeOOH^{2+} + H^{+}) \rightarrow Fe^{2+} + 2\,H^{+} + O_2^{\bullet-}$$

And, if H_2O_2 is in excess, the Fe^{2+} which is thus formed can subsequently generate ROS via the Fenton reaction. Whereas $O_2^{\bullet-}$ dismutates (via spontaneous or enzyme-catalyses reactions) to produce H_2O_2 (HENLE & LINN 1997; cf. Fig. 7):

$$2\,O_2^{\bullet-} + 2\,H^{+} \rightarrow O_2 + H_2O_2$$

The hydroxyl radical ($^{\bullet}OH$), in turn, is a highly reactive transient that can rapidly oxidise most organic and many inorganic substances (ZEPP et al. 1992, HOIGNÉ 1997). It reacts with whatever is present at its site of formation close to a diffusion-controlled rate, affecting all biomolecules, i.e. DNA, lipids and proteins (SPUHLER et al. 2010, IMLAY 2008, CABISCOL et al. 2000). Exogenous short-living ROS, such as hydroxyl radicals, damage membranes, whereas $^{\bullet}OH$ formed close to DNA (e.g. on Fe^{2+} associated with DNA) attack both DNA bases and sugars leading to strand breakage and base release (SPUHLER et al. 2010; cf. Fig. 7 and 8).

Due to the capacity of iron to generate ROS, bacteria must tightly control the uptake and storage of iron and it is not surprising that the control of iron homeostasis and responses to oxidative stress are coordinated (CORNELIS et al. 2011).

Iron uptake in bacteria is mediated by proteins which are either involved in the import of elemental Fe^{3+} by various siderophores, strong extracellular Fe^{3+} chelators, or in the direct import of Fe^{2+} (TOUATI 2000, CORNELIS et al. 2011). All genes encoding those proteins are negatively regulated by Fur (TOUATI 2000). Fur, the ferric uptake regulator, is a conserved protein in many different bacteria, which primarily operates as a repressor of iron-uptake genes by binding to a specific DNA sequence, the iron box, but can also act indirectly as a positive regulator for the biosynthesis of iron storage proteins (CORNELIS et al. 2011). Modes of gene regulation by Fur are illustrated in Figure 10.

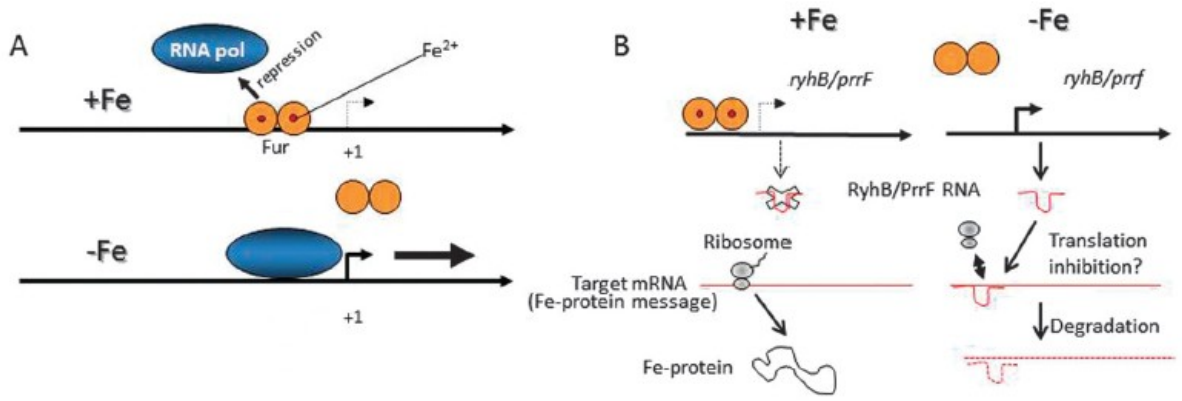

Figure 10: Regulation of iron uptake and storage by regulators belonging to the Fur family (according to CORNELIS et al. 2011). Fur, ferric uptake regulator, is a conserved protein in many different bacteria, both Gram-positive and Gram-negative, which operates primarily as a repressor of iron-uptake genes. Fur is active as a dimer, with one Fe^{2+} per monomer as a cofactor and a structural zinc site essential for activity. It binds to DNA at a specific sequence, the iron box, and acts as a transcription repressor of genes encoding proteins involved in the import of elemental Fe^{3+} by various siderophores or the direct import of Fe^{2+} (A). Fur-Fe^{2+} also represses the transcription of a small RNA (RyhB in *E. coli*, PrrF in *P. aeruginosa*) which normally destabilizes the mRNA for iron storage proteins, thereby acting indirectly as a positive regulator (B). In iron scarcity, which results in Fur inactivation, the genes are de-repressed.

Inactivation of *fur*, therefore simultaneously leads to unrestrained iron uptake and diminished incorporation of internalised iron into iron proteins (CORNELIS et al. 2011). Such a loss of iron regulation leads to oxidative stress and consequent deleterious effects, as demonstrated by a study of *E. coli fur* mutant. The mutant showed an oxygen-dependent increase in spontaneous mutagenesis, was sensitive to H_2O_2 and could not survive in the presence of oxygen if the ability to repair DNA breaks by homologous recombination was impaired (TOUATI 2000).

Moreover, similar effects were observed in *sodA sodB E. coli* mutants, which are completely devoid of cytoplasmic superoxide dismutase (SOD). They also displayed DNA breaks, increase in spontaneous mutagenesis and sensitivity to H_2O_2 (Figure 11; TOUATI 2000).

Excess in superoxide, in turn, leads to an increase in intracellular toxic iron, because $O_2^{\bullet-}$ can reduce and liberate Fe^{3+} from ferritin or liberate Fe^{2+} from iron-sulphur clusters (HENLE & LINN 1997; cf. Figure 8).

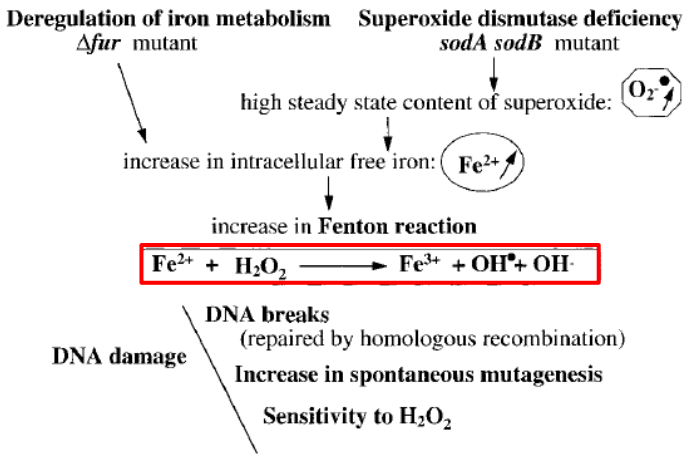

Figure 11: Similar effects of iron homeostasis deregulation and superoxide-mediated oxidative stress in *E. coli* (according to TOUATI 2000).

Furthermore, Fur regulation of SODs has been observed in several bacteria. The expression of MnSOD, for instance, is induced on Fur inactivation (iron deficiency) in *E. coli* as well as in *P. aeruginosa*, whereas FeSOD expression in *E. coli* is positively controlled by active Fur (TOUATI 2000). These opposing patterns of regulation keep global SOD activity high enough to cope with any iron environment and the related threat of oxidative stress. Besides, *fur* expression is increased in *E. coli* in response to oxidative stress (TOUATI 2000).

In summary, on the one hand, iron regulation enables cells to acquire the iron essential for survival, while maintaining low levels of free intracellular iron, which could cause oxidative stress and damage. On the other hand, iron is sensed as a signal of potential oxidative stress and defences against oxidative stress are adapted to the particular iron environment (TOUATI 2000).

1.8.3 Reactions of H_2O_2 in presence of iron(III) oxyhydroxide

Concerning the application of hydrogen peroxide in wells containing ochre, one must consider the reaction of H_2O_2 with iron oxides. A rapid decomposition of H_2O_2 in presence of granular goethite (α-FeOOH) particles happens in the form of heterogeneous catalytic reactions (LIN & GUROL 1998). A proposed reaction mechanism for H_2O_2 decomposition on iron oxide surface comprises the following reactions (LIN & GUROL 1998):

$$\equiv Fe^{III}–OH + H_2O_2 \Leftrightarrow (H_2O_2)_s \quad (1)$$

$$(H_2O_2)_s \rightarrow \equiv Fe^{II} + H_2O + HO_2^{\bullet} \quad (2)$$

$$\equiv Fe^{II} + H_2O_2 \rightarrow \equiv Fe^{III}–OH + {}^{\bullet}OH \quad (3)$$

$$HO_2^{\bullet} \Leftrightarrow H^+ + O_2^{\bullet-} \quad (4)$$

$$\equiv Fe^{III}–OH + HO_2^{\bullet}/O_2^{\bullet-} \rightarrow \equiv Fe^{II} + H_2O/OH^- + O_2 \quad (5)$$

$${}^{\bullet}OH + \equiv Fe^{II} \rightarrow \equiv Fe^{III}–OH \quad (6)$$

The mechanism involves a series of chain reactions initiated by formation of a precursor surface complex of H_2O_2 with the oxide surface, $\equiv Fe^{III}–OH$ (reaction 1). $(H_2O_2)_s$ presents the surface species of hydrogen peroxide. The dissociation of the surface complex results in the release of a peroxide radical (reaction 2). Whereas the reaction of the reduced iron with hydrogen peroxide leads to the formation of a hydroxyl radical (reaction 3). The produced radicals may react with Fe(III) and Fe(II) sites on the surface according to reactions 5 and 6. Since both peroxide and hydroxyl radicals are quite reactive, it is plausible that they will react with H_2O_2 and other species on the oxide surface before being able to diffuse back to the solution (LIN & GUROL 1998). Thus, microorganisms in the ochre may only be harmed by radical attack if they are present at the site of the radical formation.

1.9 Aim of the study

The aim of the present study was to find out, if ochreous incrustations in a water well can act as a sink and source for pathogens introduced into a well, by providing answers to the following open questions:

- Ochreous deposits in wells have a very large surface area. Could this surface be colonised by hygienically relevant bacteria?
- Are these bacteria, in contact with ochre, inactivated due to oxidative stress caused by iron released from the ochre?
- If the bacteria are not inactivated, could they integrate into the ochre matrix? Could they persist or even grow there?
- If so, will the water phase in contact with the ochre become contaminated by the bacteria?
- And, is the recommended disinfection, using hydrogen peroxide, effective against hygienically relevant bacteria attached to ochre?

2 Material and Methods

2.1 Bacterial test strains

Bacterial strains used in this study (Table 4), are type strains and were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and kept in cryogenic vials (Cryobank system, Mast Diagnostica GmbH) at -70 °C for permanent preservation.

Table 4: Bacterial test strains. Strain numbers according to American Type Culture Collection and German Collection of Microoganisms and Cell Cultures.

Species	Strain	
Escherichia coli	ATCC 11775	DSM 30083
Klebsiella pneumoniae	ATCC 13883	DSM 30104
Enterococcus faecalis	ATCC 19433	DSM 20478
Pseudomonas aeruginosa	ATCC 10145	DSM 50071
Aeromonas hydrophila	ATCC 7966	DSM 30187
Legionella pneumophila	ATCC 33152	DSM 7513

E. coli, *K. pneumoniae* and *P. aeruginosa* were cultivated on nutrient agar at 36 °C; *A. hydrophila* on nutrient agar at 30 °C. *E. faecalis* was cultivated on tryptic soy agar and *L. pneumophila* on Legionella growth medium BCYE, both at 36 °C. Cultures were stored at 4 °C, with the exception of those of *L. pneumophila*, which were kept at room temperature, and fresh cultures were prepared at least every four weeks. For inoculation of ochre, ochre suspensions and well water, fresh overnight cultures (18-22 h) were used; except for *L. pneumophila*, in which case 3 days old cultures were used.

2.2 Growth media

Media were prepared using deionised water and were autoclaved at 121 °C for 20 min, if not mentioned otherwise, and poured in portions of 25 ml into sterile Petri dishes.

Nutrient agar (NA) for microbiology, Merck

Composition (g/litre): Peptone from meat 5.0, meat extract 3.0, agar-agar 12.0; pH: 7.0 ± 0.2 at 25 °C. Preparation: 20 g nutrient agar/litre deionised water.

Tryptic soy agar, Casein-peptone soymeal-peptone agar (CASO) for microbiology, Merck

Composition (g/litre): Peptone from casein 15.0, peptone from soymeal 5.0, sodium chloride 5.0, agar-agar 15.0; pH: 7.3 ± 0.2 at 25 °C. Preparation: 40 g/litre deionised water.

Difco™ R2A Agar, BD

Composition (g/litre): Yeast Extract 0.5, proteose peptone No. 3 0.5, casamino acids 0.5, dextrose 0.5, soluble starch 0.5, sodium pyruvate 0.3, dipotassium phosphate 0.3, magnesium sulfate 0.05, agar 15.0; pH 7.2 ± 0.2 at 25 °C. Preparation: 18.2 g/litre deionised water.

Lactose TTC Agar with Tergitol® 7 for microbiology, Merck

Composition (g/litre): Lactose 20.0, peptone 10.0, yeast extract 6.0, meat extract 5.0, bromothymol blue 0.05, Tergitol®7 0.1, agar-agar 12.7. Additive: TTC 0.025. pH: 7.2 ± 0.2 at 25 °C. Preparation: 53.9 g/litre deionised water, dissolved and autoclaved. Medium was cooled in a water bath to 45-50 °C; 5 ml of a sterile filtrated 0.05 % aqueous solution of TTC were added to 100 ml basal medium, mixed homogeneously and poured into Petri dishes.

Chromocult® Enterococci Agar for microbiology, Merck

Composition (g/litre): Peptones 10.0, sodium chloride 5.0, sodium azide 0.2, dipotassium hydrogenphosphate 3.4, potassium di-hydrogenphosphate 1.6, ox bile 0.5, Tween® 80 1.0, chromogenic-mixture 0.25, agar-agar 11.0; pH 7.0 ± 0.2 at 25 °C. Preparation: 33.0 g /litre deionised water, dissolved by heating in a boiling water bath. Stirred occasionally to assist the contents dissolution (approx. 45 minutes), cooled to 45-50 °C and poured into Petri dishes. Do not autoclave! Do not overheat!

Pseudomonas selective agar (CN), Oxoid

Composition (g/litre): Gelatine peptone 16.0, casein hydrolysate 10.0, potassium sulphate 10.0, magnesium chloride 1.4, agar 11.0; pH 7.1 ± 0.2 at 25°C; Pseudomonas CN selective supplement (mg/litre): Cetrimide 200.0, sodium nalidixate 15.0. Preparation: 48.4 g/litre deionised water, 10 ml of glycerol were added, dissolved by heating in a boiling water bath and autoclaved. To one litre of agar base cooled to 50 °C contents of two vials of Pseudomonas CN Supplement, dissolved in 2 ml of a 1:1 (vol/vol) mixture of ethanol and sterile deionised water, were added, mixed homogeneously and poured into Petri dishes.

Ampicillin-dextrin agar, according to HAVELAAR et al. 1987

Composition (g/litre): Bacto Agar 15.0, dextrin from potato starch 10.0, Bacto Tryptose 5.0, sodium chloride 3.0, yeast extract granulated 2.0, potassium chloride 2.0, magnesium sulphate heptahydrate 0.2, iron(III)chloride hexahydrate 0.1. Preparation: Components of the medium were dissolved in one litre of deionised water. 8 ml of bromothymol blue solution (10 mg/ml) was added and pH was adjusted to 8.0 ± 0.2 at 25°C with NaOH solution (1 M). Medium was

autoclaved and cooled to 55°C. 10 mL freshly prepared, filter sterilized ampicillin solution (1 mg/ml) and 10 mL of sodium deoxycholate solution (10 mg/ml) were added, mixed homogeneously and poured into Petri dishes.

Legionella growth medium BCYE, ready prepared plates, Oxoid

Composition (g/litre) (ISO 11731:1998 (E)): Yeast extract 10.0, agar 12.0, activated charcoal 2.0, alpha-ketoglutarate, monopotassium salt 1.0, ACES buffer (N-2-actetamido-2-aminoethanesulfonic acid) 10.0, potassium hydroxide (KOH) 2.8, L-cysteine hydrochloride monohydrate 0.4, iron(III)pyrophosphate 0.25, deionised water ad 1000 ml.

Legionella selective medium GVPC, ready prepared plates, Oxoid

Composition (g/litre) (ISO 11731:1998 (E)): Yeast extract 10.0, agar 12.0, activated charcoal 2.0, alpha-ketoglutarate, monopotassium salt 1.0, ACES buffer (N-2-actetamido-2-aminoethanesulfonic acid) 10.0, potassium hydroxide (KOH) 2.8, L-cysteine hydrochloride monohydrate 0.4, iron(III)pyrophosphate 0.25, ammonium-free glycine 3.0, polymyxin B sulfate 80.000 iu, vancomycin hydrochloride 0.001, cycloheximide 0.08, deionised water ad 1000 ml.

2.3 Commercially available kits

Table 5: Commercially available kits used in this study.

Kit	Manufacturer
API® 20 E™	bioMérieux
API Suspension Medium	
Mineral oil	
TDA	
JAMES	
VP 1 + VP 2	
API® 20 NE	bioMérieux
API NaCl 0.85 % Medium	
Mineral oil	
JAMES	
NIT 1 + NIT 2	
Zn reagent	
Colilert®-18/Quanti-Tray®/2000	IDEXX
Enterolert®-DW/Quanti-Tray®/2000	
Pseudalert®/Quanti-Tray®/2000	IDEXX
Legionella Latex Test	OXOID
GeneMATRIX Soil DNA Purifcation Kit	EURx, roboklon
Quant-iT™ PicoGreen® dsDNA Reagent Kit	Invitrogen, Molecular Probes
iQ-Check™ Quanti *L. pneumophila* Kit	BioRad

2.4 Chemicals

Table 6: Chemicals used in this study.

Chemical	Specification	Manufacturer
Ammonium nitrate	ultrapure	Appli Chem
Ampicillin sodium salt	91.0 - 102.0 %	Appli Chem
Bacto™ Agar		BD
Bacto™ Tryptose	Enzymatic Digest of Protein	BD
Bromothymol blue sodium salt	indicator water-soluble ACS	Merck
Calcium chloride dihydrate	>99%	Fluka
Calcium nitrate tetrahydrate	>99%	Fluka
Calcium sulfate dihydrate	≥99%	Sigma
Catalase from bovine liver	lyophilized powder, ≥10,000 units/mg protein	Sigma
DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride	≥98%	Sigma Aldrich
Deoxycholic acid sodium salt	>98.0	Fluka
Dextrin from potato starch	for microbiology	Fluka
Disodium hydrogen phosphate dihydrate	for analysis	Merck
Disodium hydrogen phosphate heptahydrate	for analysis	Merck
Ethanol	99.8%	VWR
Ethylenediaminetetraacetic acid tetrasodium salt dihydrate	99.0-102.0%	Sigma
Formamide, deionized	≥99.5 %, RNAse / DNAse-free	Roth
Glycerine	99.5%, analytical reagent, redistil-led	VWR
Hydrogen peroxide 30%	stabilized, suitable for use as excip-ient EMPROVE® exp Ph Eur,BP,USP	Merck
Iron(III) chloride hexahydrate	for analysis	Merck
Magnesium sulfate heptahydrate	>99%	Fluka
Oxalic acid	anhydrous for synthesis	Merck
Paraformaldehyde	ultrapure	Merck
Potassium chloride	for analysis	Merck
Potassium dihydrogen phosphate	for analysis	Merck
Potassium nitrate	for analysis	Merck
Potassium permanganate solution	for 1000 ml, c($KMnO_4$) = 0.002 mol/l (0.01 N) Titrisol®	Merck
Potassium permanganate solution	for 1000 ml, c($KMnO_4$) = 0.02 mol/l (0.1 N) Titrisol®	Merck
Sodium chloride	99.9%	AnalaR NORMAPUR VWR
Sodium dithionite	for analysis	Merck
Sodium dodecyl sulphate, SDS	≥99.0%	Sigma
Sodium hydrogen carbonate	for analysis	Merck
Sodium pyrophosphate decahydra-te	≥95%	Sigma Life Science
Sulfuric acid	for analysis, ca. 96% solution in water	Fisher Scientific
TRIS	≥99.9 %	Roth
Trisodium citrate dihydrate	for analysis	Merck
TTC, 2,3,5-Triphenyltetrazolium chloride	for microbiology	Merck
Tween® 80	ultrapure	Roth
Water for molecular biology	nuclease-free, steam sterilised, DEPC treated water	Roth
Water ROTIPURAN®	low organic	Roth
Yeast extract granulated	for microbiology	Merck

2.5 Equipment

Table 7: Equipment used in this study.

Equipment	Manufacturer
Analytical scales, BP 210 S, max. 210 g, d=0.1 mg	Sartorius
Analytical scales, BP1200, max. 1200 g, d=0.01 g	Sartorius
Bactident® Oxidase Test Strips	Merck
Black polycarbonate membrane filters, pore size 0.2 µm	Millipore
Bottle Top Filter OR, non-fiber releasing membrane, surfactant free cellulose acetate pore size 0.20 µm	NALGENE®
Capillary tubing PTFE ID 1.1 mm	Amersham Biosciences AB
Centrifuge 5415 D	Eppendorf
Centrifuge HERAEUS Biofuge fresco	Heraeus Instruments
Centrifuge HERAEUS Fresco 21	Thermo Sientific
Centrifuge HERAEUS Pico 21	Thermo Sientific
Centrifuge, Sorvall® RC26PLUS	Sorvall
Column C 10/10	GE Healthcare
Cuvettes PMMA semi-micro, 12.5 × 12.5 × 45 mm	BRAND
Diagnostic microscope slides epoxy-coated 8-well 6 mm	Thermo Scientific, Menzel GmbH & Co KG
Epifluorescence microscope: Leitz Laborlux S, Objectives: PL Fluotar 100x / 1.32 oil, Eye-piece: 2x Periplan 10x / 18, UV-unit HBO 50	Leitz
Fluorescence spectrometer SFM25	Bio-Tek Kontron Instruments
Homogeniser Precellys 24	bertin Technologies, peqlab
Hybridisation oven	Thermo electron cooperation
Incubator ICE 400-800	MEMMERT GmbH+Co. KG
Incubator Kelvitron® t	Heraeus
Membrane filters Isopore™, 0.2 µm GTBP, black	Millipore
Membrane filters MicroPlus-31 ST, 0.45 µm pore size, Ø 50 mm, mixed cellulose ester, black	GE Healthcare Life Sciences Whatman™
Membrane filters, 0.45 µm pore size, Ø 47 mm, mixed cellulose ester, white, gridded	Pall Life Sciences
Membrane filtration apparatus, six-fold stainless-steel	Millipore
Membrane filtration apparatus, three-fold stainless-steel	Sartorius
Microscope Nicon Eclipse Ni H 550 L, Objectives: Plan Fluor 4x/0.13 OFN 25 WD 17.2, Plan Apo 100x/1.40 oil OFN 25, Eye-piece: CFI 10x/22, UV-unit Intenislight C-HGFIE, Camera: Nikon DS Fi 1, Software: NIS-Elements AR 4.10.01	Nikon
MQuant™ Peroxide test strips	Merck
Muffle Furnace	Heraeus
Orbital Shaker MaxQ™ 2000	Thermo Scientific
PCR plates, 96-well	BioRad
pH meter WTW (ph 549 ELP)	MultiCal®
Phase contrast microscope, Leica DM LS	Leica Microsystems
Quanti-Tray® Sealer Model 2X	IDEXX
Reaction chambers for Fluorescence in situ hybridisation	Vermicon
Syringe Filters Filtropur S plus 0.2, 0.20 µm porosity	Sarstedt
Syringe Filters Minisart® NML, cellulose acetate membrane, 17597-K	Sartorius
Syringe Omnifix 10 ml	B. Braun Melsungen AG
Thermostatic cabinet AL658G	AQUA LYTIC
Thoma counting chamber	Optik Labor
Thermal Cycler C1000 Touch, CFX 96 Real Time System	BioRad
Tubing connector	GE Healthcare

Tubing ISMATEC 2-stop color-coded, Tygon LFL, (mm) ID 2.79, Wall 0.84	IDEX Health & Science GmbH, ISMATEC
Tubing Pump ISMATEC IPC, 12 channels	IDEX Health & Science GmbH, ISMATEC
Tubing, Tygon, (mm) ID 2.4, OD 4.0, Wall 0.8	Saint Gobain Performance Plastics
Tubing, Tygon, (mm) ID 4.0, OD 5.6, Wall 0.8	Saint Gobain Performance Plastics

2.6 Detection methods

2.6.1 Total cell count

Cells in the well water samples were stained with the DNA-binding fluorescent dye DAPI (4',6-diamidino-2-phenylindole dihydrochloride) for enumeration of the cells with the help of an epifluorescence microscope. 4 ml of well water or the dilutions prepared in sterile deionized water were mixed with 1 ml of DAPI solution (25 µg/ml DAPI, Sigma, in 2 % formaldehyde; filter-sterilised through a 0.2 µm membrane filter and stored at 4 °C in the dark) and incubated for 20 min in the dark. The mixtures were then filtered through black polycarbonate membrane filters (IsoporeTM, ø 30 mm, 0.2 µm GTBP; Millipore) using a manifold vacuum stainless steel filtration module (Millipore). The filters were stored at 4 °C in sterile Petri dishes wrapped in aluminium foil. The cell counting was carried out using the 1000-fold magnification of an epifluorescence microscope with immersion oil and 100 µm x 100 µm counting grid. 20 randomly selected fields of view were counted for each filter. Results are given as cells/ml.

2.6.2 Fluorescence in situ hybridization (FISH)

2.6.2.1 Solutions and buffers for fluorescence in-situ hybridization (FISH)

Phosphate-buffered saline (PBS)

Composition in g/l: sodium chloride 8.00, potassium chloride 0.20, disodium hydrogen phosphate dihydrate 1.81, potassium dihydrogen phosphate 0.24.

The components were dissolved in Rotipuran® water (Roth). The pH was 7.2 ± 0.2. The solution was autoclaved for 20 min at 121 °C.

Paraformaldehyde solution (4 %)

Composition in g/l: 4 g paraformaldehyde (Merck) were dissolved in 100 ml PBS (pH 7.2) and stirred with a magnetic stirrer for 1 h at 50 °C. The solution was filter sterilized (pore size 0.2 µm).

5 M NaCl solution

292.2 g sodium chloride were dissolved in 1 l of Rotipuran® water (Roth). The solution was autoclaved for 20 min at 121 °C.

10 % (w/v) SDS

10 g sodium dodecyl sulphate (SDS, Sigma) were dissolved in 100 ml Rotipuran® water (Roth). The solution was filter sterilized (pore size 0.2 µm).

0.25 M EDTA

104.05 g ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA, Sigma) were dissolved in 1 l of Rotipuran® water. The solution was autoclaved for 20 min at 121 °C.

Hybridisation buffers

Table 8: Hybridisation buffers for oligonucleotide probes targeting bacterial 16S rRNA.

Probe	Colinsitu	Kpn	Efs 130	Psae 16S-182	AERBOMO	LEGPNE1
NaCl	0.9 M	0.9 M	0.9 M	0.9 M	0.9 M	0.9 M
Tris, pH 7.0	-	20 mM	-	-	-	-
Tris, pH 7.2	-	-	-	-	20 mM	-
Tris, pH 7.6	-	-	-	-	-	20 mM
Tris, pH 8.0	20 mM	-	20 mM	20 mM	-	-
SDS	0.01 %	-	0.01 %	0.01 %	0.01 %	0.01 %
Formamide	20 %	30 %	35 %	40 %	35 %	25 %

Washing buffers

Table 9: Washing buffers for oligonucleotide probes targeting bacterial 16S rRNA. For the preparation of hybridisation buffers, Rotipuran® water was used.

Probe	Colinsitu	Kpn	Efs 130	Psae 16S-182	AERBOMO	LEGPNE1
NaCl	250 mM	0.9 M	80 mM	56 mM	40 mM	160 mM
Tris, pH 7.0	-	20 mM	-	-	-	-
Tris, pH 7.2	-	-	-	-	20 mM	-
Tris, pH 7.6	-	-	-	-	-	20 mM
Tris, pH 8.0	20 mM	-	-	20 mM	-	-
SDS	0.01 %	0.05 %	0.01 %	0.01 %	0.01 %	0.01 %
EDTA	-	-	5 mM	5 mM	5 mM	5 mM

Oligonucleotide probes

Table 10: Oligonucleotide probes used in the present study. All probes were 5'-Cy3-labelled, HPLC cleaned and obtained as lyophilisates from Eurofins MWG Operon, Germany.

Probe	Sequence (5'-3')	Target	Reference
Colinsitu	GAG ACT CAA GAT TGC CAG TAT CAG	*E. coli*	Regnault et al. 2000
Efs 130	CCC TCT GAT GGG TAG GTT	*E. faecalis*	Meier et al. 1997
Psae 16S-182	CCA CTT TCT CCC TCA GGA CG	*P. aeruginosa*	Wellinghausen et al. 2005
LEGPNE1	ATC TGA CCG TCC CAG GTT	*L. pneumophila*	Grimm et al. 1998
Kpn	CCT ACA CAC CAG CGT GCC	*K. pneumoniae*	Kempf et al. 2000
AERBOMO	CTA CTT TCC CGC TGC CGC C	*A. hydrophila*	Bomo et al. 2004, Kämpfer et al. 1996

Oligonucleotide probe solutions

Lyophilisates were dissolved in water for molecular biology (Roth) to a final concentration of 1 µg/µl. Then stock solutions of the specific probes were prepared by diluting the dissolved lyophilisates with water for molecular biology to a final concentration of 50 ng/µl.

For preparation of the working solutions, stock solutions were diluted 1:10 in the appropriate hybridisation buffer.

DAPI solution (1 µg/ml) in PBS

40 µl of the DAPI stock solution (25 µg/ml) were mixed with 960 µl PBS.

2.6.2.2 FISH procedure

Target organisms in well water samples were detected by fluorescence in situ hybridization. For this purpose 2 ml of the water samples were centrifuged (Biofuge fresco, 5 min, 16060 x g, 4 °C), the supernatant was discarded and cells were fixed with 4 % paraformaldehyde in PBS for at least 1 h at 4 °C in the dark. Paraformaldehyde was removed by centrifugation (same conditions as above) and cells were washed in PBS by re-suspension and centrifugation and stored in 1:1 (vol/vol) ethanol/PBS at – 20 °C.

10 µl of the fixed samples were pipetted onto 8-well-slides and heat dried (40 °C or 46 °C depending on the hybridisation temperature) in the dark. The samples were dehydrated by dipping them into 50 %, 80 % and 96 % ethanol (vol/vol) for 3 min, respectively. After air-drying the slides, 10 µl of an oligonucleotide probe solution with a probe concentration of

5 ng/µl were added per well. For hybridization, the slides were incubated for 1.5 h at 40 °C (Kpn probe) or 46 °C in a humid chamber (Vermicon reactors with hybridization buffer added) in the dark. Afterwards, the slides were transferred to reaction chambers containing 25 ml pre-heated (40 °C or 46 °C) washing buffer and incubated at 40 °C (Kpn probe) or 46 °C for 20 min in order to remove unbound probe. Then the slides were washed in ice-cold deionized water, air dried and cells were counterstained by adding 10 µl of DAPI (1 µg/ml) to each well and incubating for 20 min at room temperature in the dark. Finally, the slides were once again washed in ice-cold deionized water, air dried and stored at 4 °C until enumeration.

Cells were counted using an epifluorescence microscope at 1000-fold magnification. 20 randomly selected fields of view were enumerated for each sample with the help of a counting grid (100 µm x 100 µm). The percentage of FISH-positive bacteria in relation to the number of DAPI stained cells was calculated and the concentration of FISH-positive bacteria was calculated from the total cell count determined using the DAPI method

2.6.3 Ochre dissolution experiments to allow for total cell count in ochre suspensions

2.6.3.1 Oxalic acid

A bacterial (*E. coli*) suspension was prepared in deionised water. Aliquots of the bacterial suspension were used to spike samples of ochre suspensions (0.11 g ochre wet mass/ml; final *E. coli* concentration: 10^6 cells/ml). A DAPI solution (25 µg/ml DAPI, Sigma, in 2 % formaldehyde; filter-sterilised through a 0.2 µm membrane filter and stored at 4 °C in the dark) was either added to the spiked ochre suspension or the *E. coli* cells were DAPI stained (20 min in the dark) before adding them to the ochre suspension. *E. coli* suspensions (10^6 cells/ml) in deionised water were also DAPI stained and used as controls in the dissolution experiments to test if the chemicals added to dissolve the ochre affect the cells. After treatment of the bacterial suspensions with the different dissolution agents, the mixtures were dealt with as described in section 2.6.1.

A 10 % stock solution of oxalic acid was prepared in particle-free deionised water (filtered through a cellulose acetate filter, pore size 0.2 µm; autoclaved for 20 min at 121 °C). The stock solution (pH 0.6) was diluted with deionised water to gain a 1 % (pH 1.2) and a 0.1 % (pH 2.0) solution. Aliquots of *E. coli* spiked suspensions (either 0.5 ml or 2 ml) were treated with equal amounts of the three different oxalic acid solutions (final concentration: 0.05 %, 0.5 % or 5 % oxalic acid). The contact time was either 10 min or 30 min.

2.6.3.2 Bicarbonate-buffered sodium dithionite

Bacterial (*E. coli*) suspensions (10^6 cells/ml) were prepared in ochre suspensions (0.11 g ochre wet mass/ml) and in deionised water (control samples).

Solutions (0.1 M) of sodium dithionite and of sodium hydrogen carbonate were prepared in deionised water. Equal volumes (10 ml or 25 ml) of these solutions were mixed and 1 ml of the bacterial suspensions was added. The mixtures were stirred (20 min or 5 min) and then filtered through black polycarbonate membrane filters (IsoporeTM, ø 30 mm, 0.2 µm GTBP; Millipore) using a manifold vacuum stainless steel filtration module (Millipore). 1 ml of DAPI solution (5 µg/ml) was added onto the filters in the filtration module. After an incubation time of 20 min the DAPI solution was filtered off. The examination of the filters was carried out using the 1000-fold magnification of an epifluorescence microscope with immersion oil.

2.6.3.3 Bicarbonate-buffered sodium dithionite-citrate system

Bacterial (*E. coli* or *P. aeruginosa*) suspensions (10^6 cells/ml) were prepared in ochre suspensions (0.11 g ochre wet mass/ml) and in deionised water (control samples). To 1 ml of the bacterial suspensions 40 ml of a trisodium citrate dehydrate solution (0.3 M) and 5 ml of a sodium hydrogen carbonate solution (1 M) were added. The mixture was stirred (either at room temperature or at 40 °C) and 1 g of sodium dithionite powder was added. Stirring was continued for 30 min. Afterwards the mixture was filtered through black polycarbonate membrane filters (IsoporeTM, ø 30 mm, 0.2 µm GTBP; Millipore) using a manifold vacuum stainless steel filtration module (Millipore). 1 ml of DAPI solution (5 µg/ml) was added onto the filters in the filtration module. After an incubation time of 20 min the DAPI solution was filtered off. The examination of the filters was carried out using the 1000-fold magnification of an epifluorescence microscope with immersion oil.

2.7 Quantitative real-time polymerase chain reaction (qPCR)

L. pneumophila and *P. aeruginosa* in native ochre and well water samples, as well as in the inoculated samples from the microcosm experiments, were quantified by quantitative real-time polymerase chain reaction (qPCR). For that purpose a CFX96 Touch™ Real-Time PCR Detection System (BioRad) and the CFX Manager™ Software (BioRad) was used.

2.7.1 DNA extraction and quantification

For DNA isolation and purification from ochre and well water samples, a commercially available kit (GeneMATRIX Soil DNA Purification Kit; EUR_X, roboklon) was used. The following amounts or volumes of the samples were applied in the procedure: Native ochre samples, about 0.25 g ochre wet mass (≈ 0.1 g dry mass); ochre suspensions from microcosm experiments, 2 ml ochre suspension (≈ 0.1 g ochre dry mass) and well water, 5 ml. To add the ochre from the ochre suspensions to the bead tubes (2nd step of the manufacturer's protocol), the ochre particles were extracted from the suspensions by centrifugation (Biofuge, 5 min, 16060 x g, 4 °C). The cells from the well water samples were collected by filtration (mixed cellulose ester membrane filters, 0.45 µm pore size, Ø 47 mm, white, gridded; Pall Life Sciences), the filters were cut in pieces and the pieces were added to the bead tubes.

DNA extraction was performed according to the manufacturer's protocol. For cell disruption (5th step of the protocol) a Precellys 24 homogenizer (bertin Technologies, peqlab) was used (40 s, 4800 rpm). DNA was eluted from the spin-column (22nd step of the protocol) by addition of 60 µl of elution buffer and was stored at – 20 °C until qPCR analysis.

To employ defined amounts of DNA in the qPCR analysis, and to prepare the DNA standards for *P. aeruginosa*-qPCR, DNA concentrations were determined using a commercially available kit (Quant-iT™ PicoGreen® dsDNA Reagent Kit, Invitrogen, Molecular Probes) according to the manufacturer's instructions and a spectrofluorometer (Fluorescence spectrometer SFM25, Bio-Tek Kontron Instruments) set to an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

2.7.1.1 Calculation of the Z factor

In order to quantify the genome units (GU) contained in a specified volume or mass of a sample from the starting quantity of GUs detected in 5 µl of the DNA extract during the qPCR run, one has to calculate the fraction of the processed sample that is actually analysed during the final detection. The fraction of the sample deposited in each PCR well is represented by the so-called Z factor. This value depends on the used DNA extraction protocol and at each stage in the protocol (concentration, elimination, etc.) the remaining fraction with respect to the initial sample has to be calculated.

For the GeneMATRIX Soil DNA Purification Kit (EUR_X, roboklon) the Z factor is calculated as follows:

$Z = Z_1 * Z_2 * Z_3 = 1.5 * 1.33 * 12 = 24$

With factors Z_1, Z_2 and Z_3 resulting from the following processing steps:

DNA extraction protocol

Step 6: 400 µl of the supernatant (V = 600 µl) is processed → $Z_1 = 600/400 = 1.5$

Step 9: 600 µl of the supernatant (V = 800 µl) is processed → $Z_2 = 800/600 = 1.33$

Step 22: DNA is eluted by adding 60 µl of Elution buffer = volume DNA extract

qPCR

5 µl out of 60 µl DNA extract are deposited in each PCR well → $Z_3 = 60/5 = 12$

2.7.1.2 Real-time PCR quantification of *L. pneumophila*

L. pneumophila was quantified by real-time PCR by means of the iQ-Check™ Quanti *L. pneumophila* Kit (BioRad). The kit contains all reagents required in order to perform the analysis of the samples: PCR amplification solutions including Taq DNA Polymerase and internal control, specific fluorescent probes and primers, negative control and standards for quantification. The set of four standards corresponds to a DNA concentration range of 1.5×10^1 to 4.0×10^4 GU per assay (volume: 5 µl). The probe that binds to the target sequence of *L. pneumophila* is labelled with the FAM fluorophore that emits fluorescence only when hybridized to the amplicons. The synthetic internal control DNA allows detection of any possible inhibition phenomena of the amplification reaction. It is amplified at the same time with the same primers as the target sequence of *L. pneumophila*, but is detected by a probe marked with a different fluorophore (HEX Channel). The PCR mix preparation, running of the amplification reaction, data analysis and results interpretation were all performed according to the manufacturer's instructions. The thermo-protocol used in *L. pneumophila* qPCR is given in Table 11.

Table 11: Thermo-protocol used in *L. pneumophila* qPCR.
[(a)]UDG inactivation, [(b)]Hot-start polymerase activation, [(c)]Collect data.

Cycle	Step	Temperature	Time [min]
1[(a)]		50	02:00
2[(b)]		95	15:00
3 – 52	1	95	00:15
	2[(c)]	55	00:30
	3	72	00:30

2.7.1.3 Real-time PCR quantification of P. aeruginosa

P. aeruginosa qPCR was performed according to the TaqMan probe assay described by FRÖSLER (2011) and published in TEWES (2012). In brief, a PCR reaction mix was prepared by mixing VeriQuest™ Probe qPCR Master Mix, primers and probe solutions, BSA solution and molecular-grade water in a volume ratio given in Table 12.

Table 12: PCR reaction mix for each sample in *P. aeruginosa* qPCR.

Reagent	Manufacturer	Final concentration	Volume [µl]
VeriQuest™ Probe qPCR Master Mix (2X)	USB® Products Affymetrix, Inc.	1X	25
Forward primer Pa23FP	Eurofins Genomics	300 nM	1.5
Reverse primer Pa23RPb	Eurofins Genomics	300 nM	1.5
TaqMan probe Pa23FAM	Eurofins Genomics	200 nM	1
Bovine serum albumin (BSA)	New England Biolabs	200 µg/ml	1
Water for molecular biology	Roth	-	15
Template DNA / Negative control / Standard	-	-	5

The oligonucleotides used for real-time PCR quantification of *P. aeruginosa*, forward primer Pa23FP, reverse primer Pa23RPb and TaqMan probe Pa23FAM, are specified in Table 13. Stock solutions (100 pmol/µl) of the primers Pa23FP and Pa23RPb, and the TaqMan probe Pa23FAM were stored at -20 °C in the dark.

Table 13: Sequences of oligonucleotides. Pa23FP: forward primer, Pa23RPb: reverse primer, Pa23FAM: TaqMan probe.

Oligoname	Sequence	Reference
Pa23FP	5'-TCCAAGTTTAAGGTGGTAGGCTG-3'	SCHWARTZ et al. 2006, VOLKMANN et al. 2007
Pa23RPb	5'-ACCACTTCGTCATCTAAAAGACGAC-3'	VOLKMANN et al. 2007
Pa23FAM	FAM-5'-AGGTAAATCCGGGGTTTCAAGGCC-3'-TAMRA	SCHWARTZ et al. 2006, VOLKMANN et al. 2007

For quantification, a set of five standards was prepared from a DNA extract of a *P. aeruginosa* pure culture (strain ATCC 10145). The DNA concentration of the extract was determined by means of the PicoGreen® assay (cf. section 2.7.1). The DNA extract was then serially diluted to obtain standards in the range of 3.5 pg/ml to 35.3 ng/ml. In order to get final results comparable to *L. pneumophila* qPCR, the units of the standards had to be converted to genome units (GU). According to FRÖSLER (2011) a theoretical genome weight of *P. aeruginosa* of 6.43 fg was used for the calculation. Based on this value, the concentrations of the standards corresponded to a range of 2.7 GU to 2.7×10^4 GU per assay (volume: 5 µl).

For running the amplification reaction, 45 µl of PCR reaction mix was provided per PCR well and 5 µl of either template DNA, negative control or standard were added. The thermo-protocol used in *P. aeruginosa* qPCR is given in Table 14.

Table 14: Thermo-protocol used in *P. aeruginosa* qPCR.
[a]UDG inactivation, [b]Hot-start polymerase activation, [c]Collect data.

Cycle	Step	Temperature	Time [min]
1[a]		50	02:00
2[b]		95	10:00
3 – 52	1	95	00:15
	2[c]	60	01:00

2.7.1.4 Calculation of L. pneumophila and P. aeruginosa concentration from qPCR results

The values given in the report of the CFX Manager™ Software after qPCR run correspond to the initial quantity of *L. pneumophila* or *P. aeruginosa* genome units (GU) present in 5 µl of the DNA extract. To obtain GU/ml well water sample or GU/g ochre dry mass, the following formula had to be applied:

$$X = (SQ * Z * D)/V \text{ or } M$$

X: *L. pneumophila* or *P. aeruginosa* genome units (GU) per ml or g dry mass

SQ: Starting Quantity in a 5 µl assay given by the CFX Manager™ Software

Z: Z-factor as calculated for the DNA Purification Kit (cf. section 2.7.1.1)

D: Dilution factor (if the DNA has been diluted before the PCR run)

V: Volume of the well water sample used for DNA extraction

M: Ochre dry mass used for DNA extraction

2.8 Cultivation methods

The cultivation methods listed in Table 15 were applied to quantify the culturable target bacteria in ochre suspensions and well water samples, as well as the culturable heterotrophic microorganisms contained in these samples.

Table 15: Cultivation methods used in the present study.

Cultivation method	Target	According to
Spread plate method		
HPC (Heterotrophic plate count), R2A (Difco), 20 °C, 7 d	Heterotrophs	REASONER & GELDREICH 1985
Spread plate (ochre suspensions, spiked well water) or membrane filtration (well water)		
Lactose TTC Agar with Tergitol® 7, 36 ± 2 °C, 21 ± 3 h	*E. coli*, Coliform bacteria (*K. pneumoniae*)	DIN EN ISO 9308-1; Anlage 5 Teil I Buchstabe a TrinkwV 2001
Chromocult® Enterococci-Agar, 36 ± 1 °C, 24 ± 4 h and 44 ± 4 h	Enterococci (*E. faecalis*)	Liste alternativer mikrobiologischer Nachweisverfahren gemäß § 15 Absatz 1 TrinkwV 2001, Umweltbundesamt 2012
Pseudomonas selective agar (CN), 36 ± 2 °C, 22 ± 2 h and 44 ± 4 h	*P. aeruginosa*	DIN EN ISO 16266; Anlage 5 Teil I Buchstabe c TrinkwV 2001
Ampicillin-dextrin agar, 30 °C, 24 h	*Aeromonas* spp. (*A. hydrophila*)	HAVELAAR et al. 1987
Legionella selective medium GVPC, 36 ± 2 °C, 4 d and 10 d	*Legionella* spp. (*L. pneumophila*)	DIN EN ISO 11731-2; Bundesgesundheitsbl – Gesundheitsforsch – Gesundheitsschutz 2000, 43:911-915
MPN methods		
Colilert®-18/Quanti-Tray®/2000, 36 ± 1 °C, 18 – 22 h	*E. coli*, Coliform bacteria (*K. pneumoniae*)	Liste alternativer mikrobiologischer Nachweisverfahren gemäß § 15 Absatz 1 TrinkwV 2001, Umweltbundesamt 2012
Enterolert®-DW/Quanti-Tray®/2000, 41 ± 0.5 °C, 24 h	Enterococci (*E. faecalis*)	Liste alternativer mikrobiologischer Nachweisverfahren gemäß § 15 Absatz 1 TrinkwV 2001, Umweltbundesamt 2012
Pseudalert®/Quanti-Tray®/2000, 38 ± 0.5 °C, 24 h	*P. aeruginosa*	-

The spread plate methods were used to determine the heterotrophic plate count (HPC), and to quantify culturable target bacteria in suspensions of native ochres, as well as in spiked ochre and well water samples from the microcosm and column experiments (cf. sections 2.9 and 2.10). For this purpose 100 µl of undiluted samples or of decimal dilutions of ochre suspensions (0.11 g ochre wet mass/ml) and well water samples, prepared in sterile deionized water, were spread plated in duplicate on the respective agar. Colonies were counted after the particular incubation time given in Table 15 according to the references also given in this table or according to the manufacturer's instructions of the medium. Plates with colony numbers between 30 and 300 were considered for enumeration. Results are given as colony-forming units per ml (CFU/ml) and, in the case of ochre suspensions, also in CFU/g ochre dry mass. The

dry residue of the ochre samples and their loss on ignition was determined according to DIN EN 12880:2000 and DIN EN 12879:2000 (ANONYMOUS 2001d, 2001e).

Membrane filtration, on the other hand, was applied to test native well water samples for target bacteria. In that case, 100 ml of the well water sample were filtered through mixed cellulose ester membrane filters (0.45 µm pore size, Ø 47 mm, white, gridded; Pall Life Sciences) using a three-fold stainless-steel filtration module (Sartorius). The filters were transferred onto the respective medium and incubated and evaluated according to the references given in Table 15.

The MPN methods were used for ochre suspensions as well as for well water samples, both native and the inoculated ones from the microcosm and column experiments (cf. sections 2.9 and 2.10). For native samples, either 100 ml of well water or 10 ml ochre suspension (0.11 g ochre wet mass/ml) diluted with 90 ml sterile deionized water, were tested according to the manufacturer's instructions, whereas for the inoculated samples the target bacteria concentration was determined in decimal dilutions of the samples. Results are given as most probable numbers per ml (MPN/ml) and, in the case of ochre suspensions, also in MPN/g ochre dry mass.

Bacterial isolates from native ochre and well water samples were identified by means of biochemical characterisation. Coliform bacteria and *Aeromonas* spp. were identified using the API®20 E™ and the API®20 NE system (bioMérieux), respectively. The test strips were inoculated and incubated according to the manufacturer's instructions. Identification was performed via apiweb® internet platform.

2.9 Microcosm experiments

Microcosm experiments were performed to determine the recovery and the survival of hygienically relevant bacteria in different ochre suspensions over time. For some of these ochre samples the recovery and survival of target organisms were also tested in water from the respective well, the ochre sample was taken from. The bacterial test strains used in the experiments and their cultivation conditions are listed in Table 4. The experiments were performed in 100 ml Erlenmeyer flasks on an orbital shaker at 100 rpm at room temperature or at 17 °C in the dark for up to 14 days. 20 ml aliquots of homogenised ochre suspensions (0.11 g ochre wet mass/ml; prepared in sterile deionised water or in native well water) or of well water samples were spiked with one target organism at a time (final concentration of 1 x 10^6 or 10^8

cells/ml). For that purpose bacterial suspensions of overnight (18-22 h) or three days old (*L. pneumophila*) cultures, cultivated on agar plates (cf. section 2.1), were prepared in sterile, deionised water. The ochre and well water samples were not sterilised for the experiments, but used as native samples containing the autochthonous microflora. The heterotrophic plate count, as a measure of the portion of culturable bacteria present in the samples, was determined at the beginning and the end of the experiments. The target organisms were not present in the samples at the beginning of the experiments or only in very low concentrations compared to the concentration of target organisms added.

Quantification of culturable target bacteria spiked into the ochre and well water samples was performed by means of the cultivation methods given in Table 15 at the beginning of the experiments and at regular intervals over the course of 14 days.

Target bacteria in the well water samples were also quantified using fluorescence in situ hybridization (FISH). FISH procedures and probes are described in section 2.6.2.

Furthermore, *L. pneumophila* and *P. aeruginosa* genome units were quantified by real-time PCR as described in section 2.7.

2.10 Column experiments

2.10.1 Purpose, set-up and pretests

The survival of hygienically relevant bacteria attached to ochre under flow-through conditions over time was tested in column experiments. For that purpose chromatography columns (length: 10 cm, width: 1 cm, volume: 7.8 ml) were used. The columns (one per sampling date) were filled in the lower half with either native ochre (FRI-25, cf. Table 23), which was then inoculated with target organisms within the columns, or with ochre (Tw-22, cf. Table 23) already inoculated outside the columns (5 g ochre wet mass/column; corresponding to a volume of 3 ml). In the case of the experiments with inoculation of the ochre with target organisms within the columns, one column without the addition of ochre was run as a control. The columns were arranged horizontally, installed in the experimental set-up (see Figure 12) and flooded with synthetic groundwater.

The composition of the synthetic groundwater is given in Table 16. After preparation, the synthetic groundwater was filtered through a cellulose acetate filter (pore size 0.2 µm).

Table 16: Synthetic groundwater (sGW) used for the column experiments. Composition according to groundwater composition of aquifer type Sands & Gravels, lower Rhine embayement, WENDLAND et al. 2008.

Chemical compound	Final concentration [mM]	Amount [g] per 5 litre
$CaSO_4$ x 2 H_2O	0.5	0.4
$NaHCO_3$	2.0	0.8
$CaCl_2$ x 2 H_2O	0.7	0.5
KNO_3	0.1	0.05
$MgSO_4$ x 7 H_2O	0.6	0.7
$Ca(NO_3)_2$ x 4 H_2O	0.25	0.3
NH_4NO_3	0.01	0.005

The experiments were run under continuous flow-through of fresh synthetic groundwater which was pumped from storage vessels through the columns into effluent vessels. The set-up of the column experiments is shown in Figure 12.

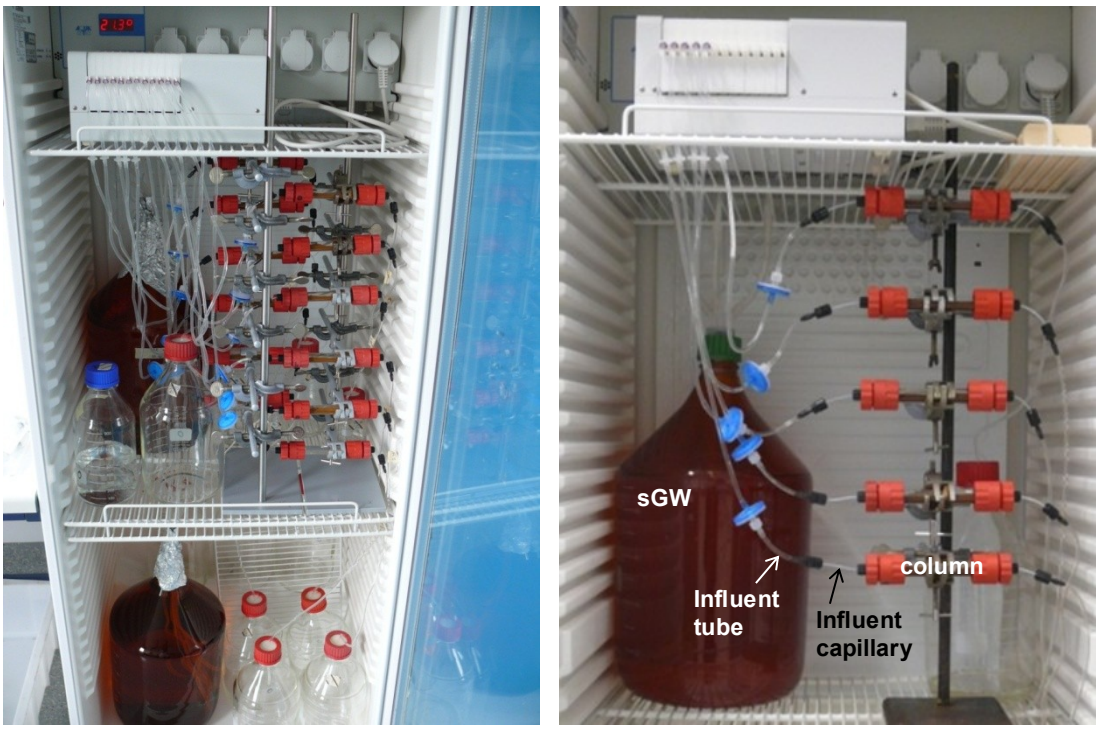

Figure 12: Set-up of a column experiment and samples tested for target organisms. Swab samples were taken from the inner surface of the control column, of the influent capillary and tube. Also the synthetic groundwater (sGW) from the storage vessel and the water phase from the column were tested.

All parts of the set-up were sterilised by autoclaving (121 °C, 20 min) before assembly under sterile conditions, except the syringe filters (Minisart® NML, cellulose acetate membrane, 17597-K) which were ETO-sterile. The system was built up in a thermostatic cabinet to control ambient temperature. The experiments were run at 17 °C.

In preliminary tests, a coloured solution ($KMnO_4$, 2 mg/ml) was injected into the columns to determine the volume needed to fill the complete space above the ochre in the columns (i.e. the volume of the water phase). After injection, the flow-through was started (flow rate: 18 ml/h) and the efflux of the potassium permanganate solution was monitored to define the time after which the water phase was exchanged once. This point was set as first sampling date.

2.10.2 Inoculation of ochre with target organisms within the columns

10 ml of a bacterial suspension (one target organism at a time, 1 x 10^8 cells/ml), prepared in synthetic groundwater, were injected into each column. The effluent was discarded and the column was connected to a sterile discharge vessel. The inoculated columns were left stagnant for 17 h or 5 h (in the case of the experiment with *L. pneumophila*). Then the continuous flow-through (flow rate: 18 ml/h) was started.

2.10.3 Inoculation of ochre with target organism outside the columns

12 ml of a bacterial suspension (one target organism at a time, 1 x 10^8 cells/ml), prepared in synthetic groundwater, were added to 6 g ochre (wet mass) in centrifuge tubes and mixed by vortexing. The suspensions in the tubes were left stagnant overnight (~ 17 h). After that, the ochre particles were collected by centrifugation (1400 x g, 5 min) and washed twice (discard supernatant, add 12 ml sGW, mix, centrifuge). Then 5 g of the inoculated ochre (wet mass) was filled into the lower half of each column (corresponding to about 2 g ochre dry mass/column). The columns were installed in the experimental set-up and flooded with synthetic groundwater. Then the continuous flow-through (flow rate: 18 ml/h) was started.

2.10.4 Experimental procedure

The ochre, which was either inoculated with hygienically relevant bacteria inside or outside the columns, as well as the water phase from the column, were then tested for target organisms after different times of flow-through. The first samples were taken after 1 h of flow-through. The water phase from the control column without ochre (only in case of inoculation within the columns) and from one column with ochre was extracted and, at this sampling date, also the effluents of these columns were sampled. The column with ochre was disassembled

and the ochre was taken from the column. Then the flow-through was started again. Samples from the columns were taken as described in regular intervals for up to 14 d.

In addition, also swab samples from different parts of the set-up were taken and suspended in sterile deionised water. An overview of all samples and a description of sample preparation are given in Table 17. An ochre suspension was prepared from the homogenised ochre sampled from the columns (0.11 g ochre wet mass/ml autoclaved deionised water). The ochre suspension, water phase samples and suspended swab samples were tested for target organisms according to the methods described in section 2.8.

Table 17: Samples tested for target organisms in the column experiments and their preparation.

Sample name	Sample preparation
Ochre	complete ochre sample from the columns; preparation of an ochre suspension (5 g ochre + 45 mL sterile deionised water)
Water phase	sampled from the columns with a syringe at the inlet of the column (~ 4 mL)
Column	swabbing of the inner surface of the column (31,4 cm^2); preparation of a suspension in sterile deionised water (2 mL) – control columns without ochre → whole surface – columns with ochre → ochre-free upper half of the column
Influent tube	swabbing of the inner surface of the longitudinally cut tube; preparation of a suspension in sterile deionised water (2 mL)
Influent capillary	swabbing of the inner surface of the longitudinally cut capillary; preparation of a suspension in sterile deionised water (2 mL)
Synthetic groundwater, sGW	sample from the storage vessel of the experimental setup

2.11 Disinfection experiments with H_2O_2

Ochre (Tw-22, cf. Table 23) spiked with either *E. coli*, or *E. faecalis*, or *P. aeruginosa* was treated with H_2O_2 in different concentrations and using different experimental procedures. The inoculation of the ochre with target organisms was performed according to the procedure described in section 2.10.3. Then the inoculated ochre was either filled into columns (c.f. section 2.10) and treated with hydrogen peroxide under stagnant or flow-through conditions in the columns, or it was kept in the centrifuge tubes (in that case only 5 g ochre wet mass were inoculated with 10 ml of a bacterial suspension initially) and the hydrogen peroxide solutions were added into the tubes.

In columns under stagnant conditions three H_2O_2 concentrations (0.15, 0.3 and 1 g/l) were tested and under flow-through conditions four (0.15, 0.3, 1 and 10 g/l). In the first case, the

columns, filled with ochre in the lower half, were flooded with the hydrogen peroxide solutions and the inlet and outlet tubes of the columns were closed by use of hose clamps. In the latter case, the hydrogen peroxide solutions of the different concentrations were pumped through the columns like the synthetic groundwater in the column experiments (cf. Figure 12). The ochre in the centrifuge tubes was either treated with 1 %, 2 % or 3 % hydrogen peroxide solution (corresponding to $c(H_2O_2)$ of 12, 23 or 34 g/l) by adding 10 ml of one of the H_2O_2 solutions to 5 g ochre wet mass (or 5 ml H_2O_2 to 2.5 g ochre wet mass; in one of the four experiments); synthetic groundwater was used as control. The ochre and aqueous solutions were not mixed. The macroscopic reactions were monitored and the numbers of culturable target bacteria, after a contact time of 24 h, were determined (cultivation methods see section 2.8).

Furthermore, the effect of hydrogen peroxide against *E. coli*, *E. faecalis* and *P. aeruginosa* in synthetic groundwater and ochre suspensions was studied. For that purpose, bacterial suspensions were prepared in sterile deionised water and 20 ml aliquots of synthetic groundwater or ochre suspensions (ochre Tw-22, cf. Table 22, in synthetic groundwater; 0.11 g ochre wet mass/ml, and, in the case of experiments with *P. aeruginosa*, also 0.2 mg ochre wet mass/ml) were inoculated with one of the target organisms respectively (1×10^8 cells/ml). Following a static incubation overnight (17 h at 17 °C), different amounts of hydrogen peroxide solutions were added, resulting in final concentrations of H_2O_2 of 0.15, 0.3, 1 and 10 g/l. After a contact time of 24 h, numbers of culturable target organisms were determined (cultivation methods see section 2.8). Additionally, different contact times (0.25, 0.5, 1, 2, 4, 6, 8 and 24 h) were tested for some of the hydrogen peroxide concentrations. To inactivate the residual hydrogen peroxide, after the respective contact time, 10 µl of a catalase solution (25 µg/µl) was added to 10 ml of the respective sample (final catalase concentration: 25 µg/ml). The catalase solution was prepared, right before its use, in synthetic groundwater (0.05 g catalase in 2 ml sGW) and filter-sterilised (Syringe Filters Filtropur S plus 0.2, 0.20 µm porosity, Sarstedt).

2.11.1 Determination of hydrogen peroxide concentration

The initial hydrogen peroxide concentration of the different solutions and the concentrations after contact with ochre suspensions or synthetic groundwater samples, each containing either *E. coli*, or *E. faecalis* or *P. aeruginosa*, were checked with test stripes (MQuant™ Peroxide test strips) and, in addition, determined by permanganometric titration after different periods of time. For titration ready-to-use potassium permanganate solutions (Merck) of $c(KMnO_4)$ of 0.002 mol/l or 0.02 mol/l were used. Titration was performed in 100 ml wide mouth Erlen-

meyer flasks: To 2 ml of sample, 1 ml of a H_2SO_4 solution (25%, v/v) and 7 ml deionised water were added; $KMnO_4$ solution was added until a permanent light pink colourisation of the solution.

The permanganometric titration is based on the following redox reaction:

Red.:	$MnO_4^- + 5\ e^- + 8\ H^+$	→	$Mn^{2+} + 4\ H_2O$	* 2
Ox.:	H_2O_2	→	$O_2 + 2\ H^+ + 2\ e^-$	* 5

6

$$2\ MnO_4^- + \cancel{10\ e^-} + \cancel{16}\ H^+ + 5\ H_2O_2 \rightarrow 2\ Mn^{2+} + 8\ H_2O + 5\ O_2 + \cancel{10\ H^+} + \cancel{10\ e^-}$$

$$2\ KMnO_4 + 3\ H_2SO_4 + 5\ H_2O_2 \rightarrow 2\ MnSO_4 + 8\ H_2O + 5\ O_2 + K_2SO_4$$

The hydrogen peroxide concentration in the samples was calculated according to the following equation:

$$c(H_2O_2) = ((c_{eq}(KMnO_4)\ [mol/l] * V(KMnO_4)\ [l] * (M(H_2O_2)\ [g/mol] / f_{eq}(H_2O_2))) / V(Sample)\ [l]$$

$c_{eq}(KMnO_4)$ $= c(KMnO_4) * f_{eq}(KMnO_4)$

$= 0.002\ mol/l * 5 = 0.01\ N$

$= 0.02\ mol/l * 5 = 0.1\ N$

$M(H_2O_2)$ $= 34.02\ g/mol$

$f_{eq}(H_2O_2)$ $= 2$

3 Results

The strategy of the present study was to simulate a contamination of a water well, containing ochreous deposits, by hygienically relevant bacteria in order to find out

- if those bacteria can persist in the ochre deposits in the well,
- if they can contaminate the water in the well from out of the ochre
- and if the recommended disinfection using hydrogen peroxide is effective against those bacteria attached to ochre.

For that purpose, ochre samples were spiked with indicator bacteria of faecal and non-faecal contamination and opportunistic pathogens, followed by analysis of the recovery, survival or growth of these bacteria. *E. coli* and *E. faecalis* (the latter belonging to the group of intestinal enterococci), *K. pneumoniae* (belonging to the group of total coliform bacteria), *P. aeruginosa*, *L. pneumophila* and *A. hydrophila* were chosen as examples of the two categories of hygienically relevant microorganisms (cf. sections 1.5.2, 1.5.3 and 1.5.4).

The water phase in contact with ochre, contaminated by the target bacteria, was analysed for the occurrence of these bacteria over the course of the experiments (up to 14 days).

Effectiveness of H_2O_2 treatment against hygienically relevant bacteria attached to ochre was studied.

3.1 Ochre and well water samples

During the project period 15 ochre samples and 13 well water samples were obtained from project partners. Six of the ochre samples and three of the water samples were from drinking water wells, whereas the other samples were from dewatering wells in opencast mines. Origin of the samples, sampling dates and designation of the samples are given in Table 18.

Sampling of the ochre and well water at the drinking water wells was carried out by project partners of the Chair Environmental Microbiology and the Chair of Water Quality Control, TU Berlin. The samples from the wells in opencast mines in Saxony were taken by project partners of the Chair of Aquatic Geomicrobiology, Friedrich-Schiller-University Jena.

Ochre samples were taken from the submersible borehole pumps or the rising pipes of the wells. The water samples were taken from the delivery flow at the well head (see Figure 13).

Table 18: Ochre and water samples. NRW, North Rhine-Westphalia; SN, Saxony; BE, Berlin.

Origin of the sample	Sampling date	Type of sample	Designation
Dewatering wells			
Opencast mine NRW	29.09.2011	Ochre	O. WR 2378
	26.09.2012	Ochre	O. W 5465
	26.09.2012	Water	W. W 5465
Opencast mine NRW	29.09.2011	Ochre	O. HS 1362
	12.06.2012	Water	W. HR 904
	12.06.2012	Water	W. HR 903
	12.06.2012	Water	W. 838
	12.06.2012	Water	W. HR 1487
	12.06.2012	Water	W. HR 1172
	12.06.2012	Water	W. HR 1173
	12.06.2012	Water	W. H 1424
	12.06.2012	Water	W. H 1426
	18.06.2012	Ochre	O. H 1424
Opencast mine SN	29.09.2011	Ochre	O. VK 24
Opencast mine SN	03.07.2012	Ochre	O. 4616
	03.07.2012	Ochre	O. NW 23A
	03.07.2012	Ochre	O. NW 63A
	27.09.2012	Ochre	O. NW 83
	27.09.2012	Water	W. NW 83
Drinking water wells BE	26.10.2011	Ochre	O. BWB
	17.01.2013	Ochre	O. Tw-21
	21.01.2013	Ochre	O. To-05
	25.01.2013	Ochre	O. FRI-25
	20.02.2013	Water	W. Tw-21
	20.02.2013	Ochre	O. Tw-24
	23.05.2013	Ochre	O. Tw-22
	27.05.2013	Water	W. To-05
	24.09.2013	Water	W. FRI-25

Figure 13: Ochre and water sampling at dewatering wells. (a) Well W 5465, submersible borehole pump just lifted out of the well. (b) Well HS 1362, extraction of the rising pipe to exchange the pump (new pump in the front, segments of the rising pipe behind it). (c) Ochre sampling from the outside of the pump. Ochre inside the check valve of the pump from (d) well W 5465 and (e) well HS 1367. (f) Sampling tap (flame-resistant) at a well, for water sampling from the delivery flow.

3.2 Establishment of the methods for quantification of all microorganisms and target bacteria in ochre samples and well water

3.2.1 Culture-independent methods

It has long been recognised that only a very small fraction of microorganisms present in a sample are detected by cultivation techniques (KÖSTER et al. 2003). For oligotrophic to mesotrophic aquatic habitats, it has frequently been reported that direct microscopic counts exceed plate counts by several orders of magnitude (AMANN et al. 1995). This phenomenon has been described by the phrase "the great plate count anomaly" (STALEY & KONOPKA 1985) and by now there is little doubt that in most cases the majority of microscopically visualised cells are viable but just do not form colonies on plates (AMANN et al. 1995). Two types of cells contribute to this silent but active majority: (1.) unknown species that have never been cultured before for lack of suitable methods and (2.) known species for which the applied cultivation conditions are not suitable or which have entered a nonculturable state (AMANN et al. 1995). Many bacteria, including a variety of important human pathogens, are known to respond to various environmental stresses by entry into the so-called 'viable but non-culturable' (VBNC) state. Bacteria in the VBNC state fail to grow on the routine bacteriological media on which they would normally grow and develop into colonies, but are still alive (OLIVER 2010). The target organisms in the present study, *E. coli*, *E. faecalis*, *K. pneumoniae*, *P. aeruginosa*, *L. pneumophila* and *A. hydrophila*, are all known to enter the VBNC state (OLIVER 2005, 2010; LI et al, 2014).

For this reason culture-independent methods should be applied alongside traditional cultivation techniques to quantify the cells present in a sample.

3.2.1.1 Total cell count

The total cell count method is applied to quantify all cells, culturable or not, in a sample by epifluorescence microscopy after staining them with the DNA-binding fluorescent stain DAPI (4',6-diamidino-2-phenylindole). For quantification of all cells in well water samples the method could be applied without any restriction. The results are given in context of the microcosm experiments to test the persistence of the target organisms in ochre suspension and well water (see section 3.5.3).

The use of the method for quantification of cells in ochre, however, is hindered by the ochre particles. Consequently, ochre and bacteria have to be separated either by detachment of bacteria from the ochre or by dissolution of the ochre itself. An extensive detachment of cells

could not be achieved by washing the ochre with deionised water and afterwards with a dispersing solution (containing tetrasodium pyrophosphate and Tween 80) as the results of the quantification of target organisms by cultivation showed. The liquid phase of the ochre suspension contained only a minor portion of the target organisms added to the ochre suspension, even after the first and the second washing step (supernatant 2 and 3), whereas the major portion was detected in the sediment of the final centrifugation step (Table 19).

Table 19: Detection of target organisms in components of spiked ochre suspensions by two different methods. Numbers (in CFU/ml or MPN/ml) detected in the whole ochre suspension were set to 100 %; numbers in the liquid phase of the suspension after separation by centrifugation (supernatant 1), after a first and a second washing step (supernatant 2 and 3) and in the remaining sediment are show in percentages of the numbers in the whole ochre suspension. LacTTC, spread plate method on Lactose TTC Agar with Tergitol® 7; Colilert, MPN-method Colilert®-18/ Quanti-Tray®/2000.

Target organism	*E. coli* [%]		*K. pneumoniae* [%]	
Detection method	LacTTC	Colilert	LacTTC	Colilert
Ochre suspension	100	100	100	100
Supernatant 1	6.8	5.9	0.2	0.4
Supernatant 2	0.4	0.2	0.04	0.02
Supernatant 3	0.1	0.04	0.02	0.01
Sediment	48.2	77.4	37.9	47.4

The microscopic examination after DAPI staining of the water phase of the centrifuged ochre suspension and the supernatants after the respective washing step revealed strong background fluorescence. It even increased during observation and a quantification of cells was not possible.

A separation of ochre particles and bacteria was also not obtained by ultrasonic treatment followed by centrifugation. The supernatants were turbid and orange coloured. DAPI stained samples of the supernatants showed high background fluorescence and only few to no cells were visible (Figure 14). Thus different methods to dissolve the ochre before performing the total cell count were tested (see section 2.6.3 and section 3.2.2).

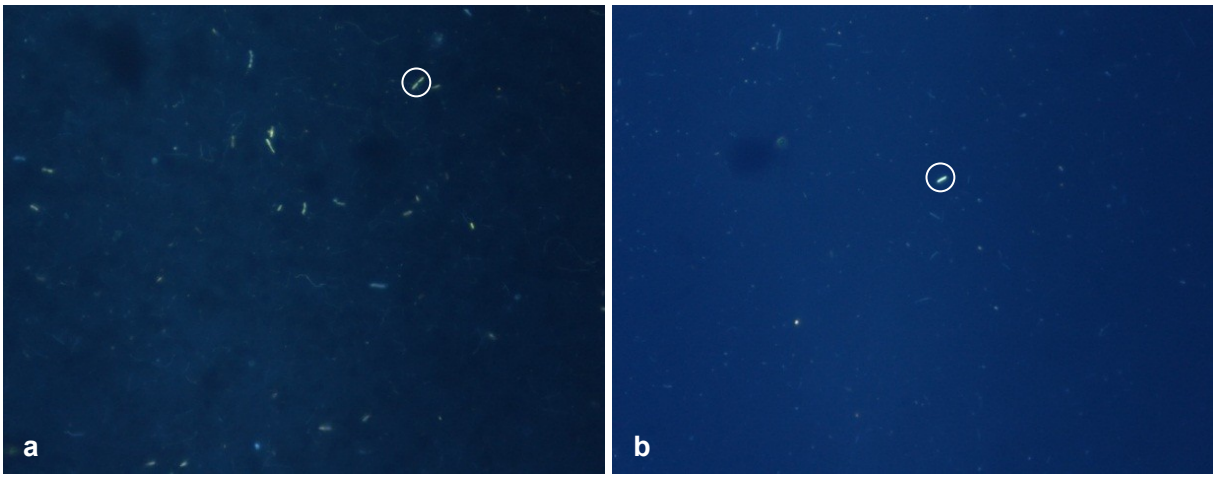

Figure 14: No separation of cells and ochre particles obtained in an ochre suspension treated by ultrasonic sound followed by centrifugation. Fluorescence micrographs of (a) the liquid phase of an ochre suspension spiked with bacteria and separated by centrifugation and (b) the supernatant of the sample after resuspension of the sediment, ultrasonic treatment and centrifugation (Original magnification: 1000x). Cells (e.g. see circles) stained with DAPI. Background fluorescence due to ochre particles.

3.2.1.2 Fluorescence in situ hybridization (FISH)

The quantification of target organisms by FISH was possible in well water samples. The results are given in context of the microcosm experiments to test the persistence of the target organisms in ochre suspension and well water (see section 3.5.4).

FISH for the quantification of *E. coli* was also performed for the water phase of an *E. coli*-spiked ochre suspension separated by centrifugation, as well as for the supernatants after a first and a second washing step (deionised water; dispersing solution) and of the resuspended sediment. But the microscopic examination of the samples revealed similar difficulties as the total cell count of the same samples. Ochre particles interfered significantly with the fluorescence microscopic quantification of the cells. Therefore, no quantification of target organisms in ochre by FISH could be achieved.

3.2.2 Dissolution of ochre

Since fluorescence microscopic quantification of cells after staining with the DNA-binding fluorescent stain DAPI or the quantification of target organisms by FISH in ochre suspensions was strongly interfered by ochre particles different methods to dissolve the ochre were tested.

In principle, three types of reactions affect the dissolution of Fe(III) oxides (HOUBEN 2003b):

- proton assisted dissolution
- ligand controlled dissolution (complexation)
- reduction

In the process of proton-assisted dissolution adsorption of protons by surface OH groups leads to a weakening of the Fe-O bond and promotes detachment of Fe from the bulk oxide (CORNELL & SCHWERTMANN 1996).

The reaction between protons and Fe(III) oxides can be written as

$$FeOOH + 3\ H^+ \rightarrow Fe^{3+} + 2\ H_2O$$

The efficiency is strongly dependent on pH, and reaches its optimum at pH << 2 (HOUBEN 2003b).

In ligand controlled dissolution ligand molecules use free electron pairs to attach themselves to the metal ion on the mineral surface. The metal complex subsequently detaches slowly from the mineral surface (HOUBEN 2003b).

The general reaction for ligand promoted dissolution may be written as follows (CORNELL & SCHWERTMANN 1996)

$$\equiv Fe^{III}\text{-}OH + L^- + H^+ \rightarrow \equiv Fe^{III}L + H_2O \rightarrow Fe^{III}L_{aq} + H_2O$$

Only bidentate ligands are effective; complexing agents may be salts of carboxylic acids such as oxalate, and complexed Fe is less susceptible to re-precipitation (HOUBEN 2003b).

Reduction processes transform very sparingly soluble Fe(III) compounds into soluble Fe(II); an example of a reducing agent is sodium dithionite, $Na_2S_2O_4$ (HOUBEN 2003b). The overall reaction can be written as

$$2\ FeOOH + S_2O_4^{2-} + 4\ H^+ \rightarrow 2\ Fe^{2+} + 2\ HSO_3^- + 2\ H_2O$$

Combinations of reducing and complexing agents are known to further enhance dissolution rates (HOUBEN 2003b)

In the present study, treatment with oxalic acid, buffered sodium dithionite, or buffered sodium dithionite in addition of citrate was applied to dissolve ochre samples (results are given in the following three sections).

Organic acids combine proton-assisted, ligand-assisted and reductive dissolution mechanisms. They form surface complexes on the Fe oxide surface where they either promote electron transfer (reduction of Fe(III) to Fe(II)) and/or detach Fe as a Fe-organic complex. Oxalic acid, the simplest dicarboxylic acid (HOOC-COOH), operates as proton-donor and ligand at the same time (HOUBEN 2003b).

Sodium dithionite ($Na_2S_2O_4$) has a high potential for Fe oxide dissolution, but since the highest reduction rates are achieved under neutral pH, buffering e.g. with bicarbonate is essential. The efficiency can be enhanced further by addition of a complexing agent, e.g. citrate (HOUBEN 2003b). This combination is a standard method e.g. in soil science (MEHRA & JACKSON 1960).

3.2.2.1 Oxalic acid

Final concentrations of 0.05 % and 0.5 % oxalic acid caused no visible reaction in ochre suspension in both volumes tested (0.5 ml or 2 ml of ochre suspension). Oxalic acid in a final concentration of 5 % led to a change in colour and turbidity of the ochre suspension. The turbid ochre suspension was altered into a clear yellow fluid. This reaction happened immediately in 0.5 ml of ochre suspension whereas the reaction took about 10 min in a volume of 2 ml. The microscopic examination of all samples, however, showed no cells but only ochre particles or a thick layer of ochre.

The modification of the experiment by either staining of cells with DAPI before mixing them with ochre and treating this suspension with oxalic acid (5 % final concentration) or by extending the contact time of oxalic acid and ochre suspension from 10 min to 30 min made no difference in the microscopic examination. In all samples the high amount of ochre particles did not allow for the quantification of cells.

3.2.2.2 Bicarbonate-buffered sodium dithionite

The addition of 1 ml ochre suspension (0.04 g dry mass/ml) to a mixture of 25 ml sodium dithionite (0.1 M) and 25 ml sodium bicarbonate (0.1 M) let to the dissolution of the ochre within minutes, but black particles were visible in the sample. DAPI-stained cells could be detected microscopically in this sample, but could not be quantified accurately due to a still considerable amount of particles. Furthermore samples of the same ochre reacted differently in different preparations of the experiment (Figure 15). The dissolution reaction seemed to be not reproducible. Also the different ochre samples tested reacted differently in contact to the bicarbonate-buffered sodium dithionite solution. Changes in colour and turbidity where detected but only one of four ochre samples tested showed a significant dissolution described above.

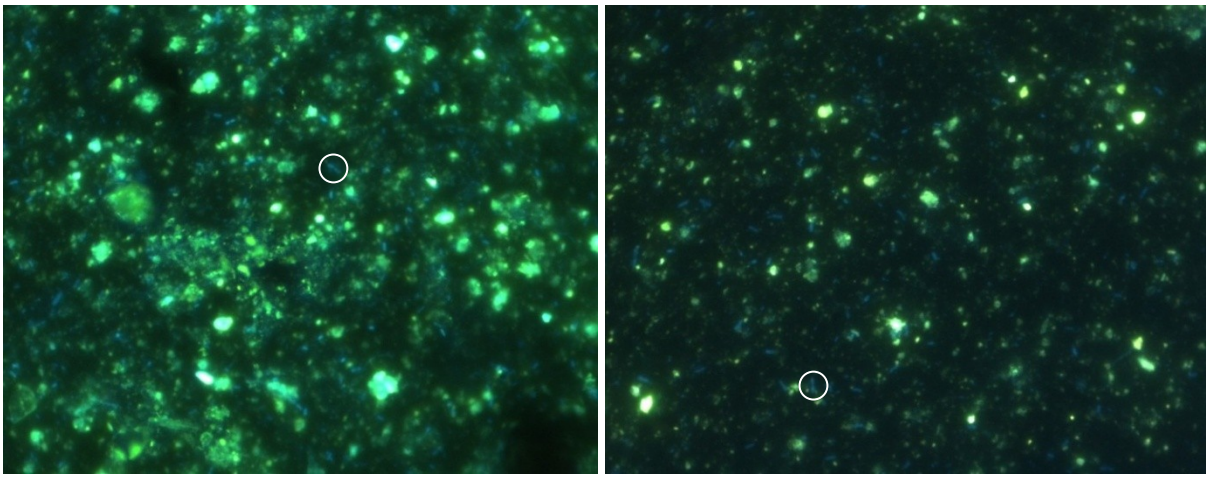

Figure 15: Ochre dissolution experiments using bicarbonate-buffered sodium dithionite. Fluorescence micrographs of suspensions of the same ochre sample, sterilised by autoclaving and then spiked with *E. coli*, after treatment with sodium dithionite (0.1 M) and sodium bicarbonate (0.1 M) and staining with DAPI (Original magnification: 1000x). The pictures show the results of two independent preparations of the experiment. DAPI-stained cells (e.g. see circles) could be detected in both preparations, but the samples also contained different amounts of autofluorescent particles interfering with the quantification of cells.

3.2.2.3 Bicarbonate-buffered sodium dithionite-citrate system

Dissolution of ochre particles in suspensions of four different ochre samples was also tried by the use of bicarbonate-buffered sodium dithionite in addition of trisodium citrate as a chelating agent. Different changes in colour and turbidity were detected for the different ochre samples, but all samples still contained considerable amounts of particles so that no definite quantification of cells were possible (Figure 16). Again samples of the same ochre reacted differently in different preparations of the experiment. The method was not reproducible.

Table 20: Summary ochre dissolution methods.

Ochre dissolution method (see page 38f.)	Reference	Result of microscopic examination of ochre sample after treatment
Oxalic acid	HOUBEN 2003b; B. Braun TU Berlin, person. comm.	High amount of ochre particles; no detection of cells or quantification of cells not possible
Bicarbonate-buffered sodium dithionite	HOUBEN 2003b	Considerable amount of particles; cells detected but quantification not possible; method not reproducible
Bicarbonate-buffered sodium dithionite-citrate system	HOUBEN 2003b; MEHRA & JACKSON 1960	Considerable amount of particles; cells detected but quantification not possible; method not reproducible

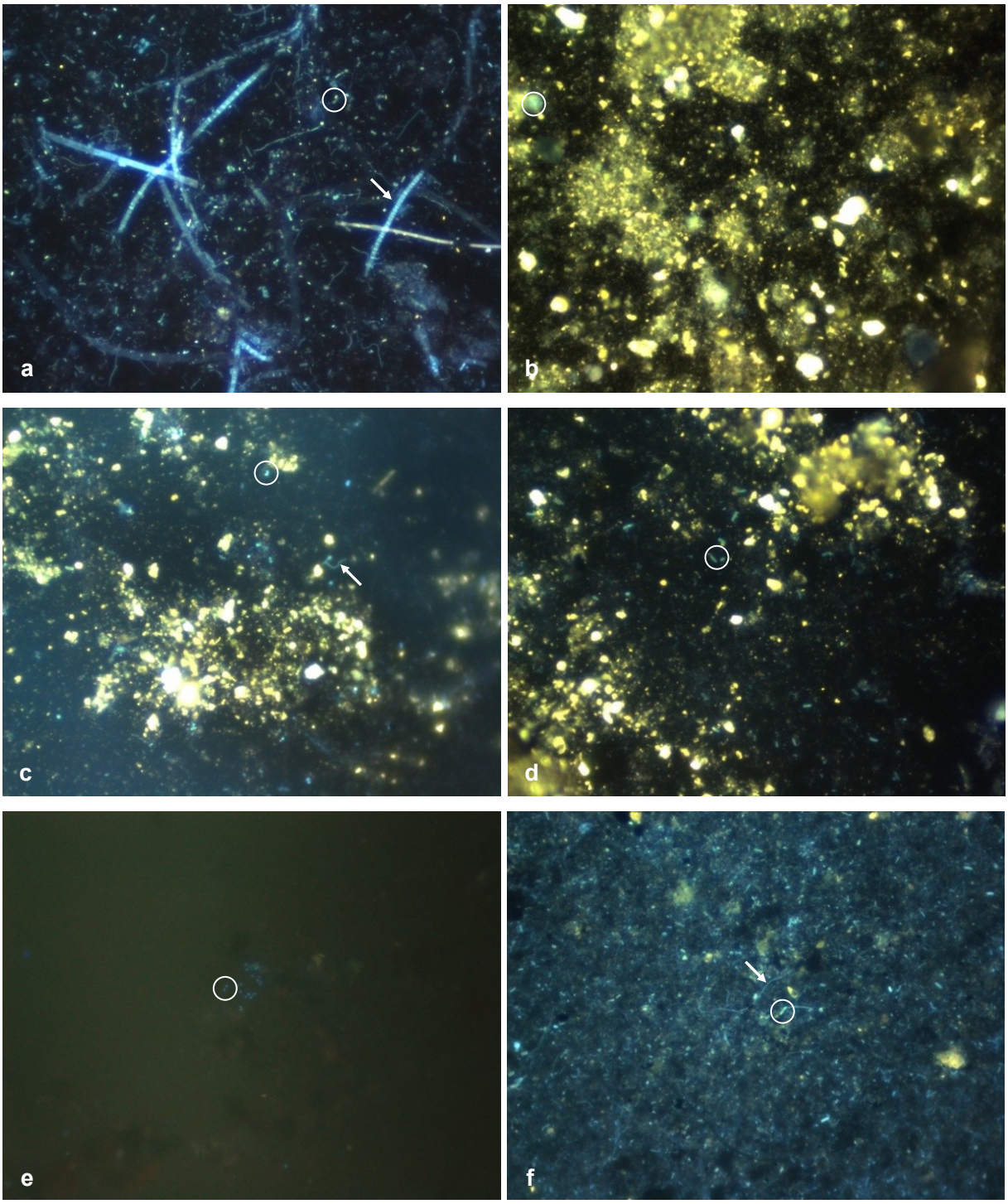

Figure 16: Ochre dissolution experiments using bicarbonate-buffered sodium dithionite in addition of trisodium citrate. Fluorescence micrographs of suspensions of three different ochre samples, HS 1362 (a, b), VK 24 (c, d) and WR 2378 (e, f), sterilised by autoclaving and then spiked with either *E. faecalis* (a, c, e) or *P. aeruginosa* (b, d, f), after treatment with bicarbonate-buffered sodium dithionite in addition of trisodium citrate and staining with DAPI (Original magnification: 1000x). The samples contained DAPI-stained cells (e.g. see circles), other DAPI-stained structures (e.g. see arrows) and more or less autofluorescent particles. Treated suspensions of the same ochre samples showed different amounts of cells, structures and particles in different preparations of the experiment.

Even though these different techniques were tried, it was not possible to establish a reproducible method for microscopic quantification of target bacteria in ochre samples. For two of the

target organisms, *L. pneumophila* and *P. aeruginosa*, the also culture-independent method of quantification by real-time polymerase chain reaction (qPCR), already established for water and plankton samples (FRÖSLER 2011), could be adopted for the quantification of these bacteria in ochre samples. The results achieved for some of the samples from the microcosm experiments are given in section 3.5.5. However, since it was not possible, due to the limited time in the project, to establish qPCR methods for the other organisms, the quantification of the target bacteria in most of the samples was done by cultivation methods. The suitability of the different cultivation techniques for the detection of the target bacteria in ochre was tested; results are given in the next section.

3.2.3 Cultivation methods

The different cultivation methods for the detection of the target organisms were checked for their suitability to quantify the respective organism in ochre suspensions. The recovery rate (ratio of CFU or MPN detected to initial cell concentration adjusted within the spiked samples) achieved for the target organisms with the respective method was also determined. For the detection and quantification of *E. coli*, *K. pneumoniae*, *E. faecalis* and *P. aeruginosa* two different methods were tested respectively, one spread plate technique using a selective agar and one MPN method.

With the exception of the MPN methods Pseudalert®/Quanti-Tray®/2000 and Enterolert®-DW/Quanti-Tray®/2000 all employed methods were suitable for the detection of target organisms in ochre suspension. Similar or even higher recovery rates of the different target organisms in ochre suspensions (spread plate methods: up to 50 mg ochre dry mass/ml, MPN methods: up to 5 mg ochre dry mass /ml) were detected as compared to the respective organism in water (well waters or deionised water) (see Table 21), the ochre particles did not interfere with the methods.

The MPN methods Colilert®-18/Quanti-Tray®/2000, Enterolert®-DW/Quanti-Tray®/2000 and Pseudalert®/Quanti-Tray®/2000 were also checked for abiotic hydrolysis of the substrates by ochre particles (up to 5 mg ochre dry mass/ml) using different ochre samples sterilised by autoclaving. For all three methods no abiotic hydrolysis was detected, but the Enterolert®-DW/Quanti-Tray®/2000 and Pseudalert®/Quanti-Tray®/2000 methods showed other drawbacks. Enterolert®-DW/Quanti-Tray®/2000, on the one hand, yielded differing results compared to the spread plate technique on Chromocult® Enterococci-Agar, when a high amount of ochre was present (5 mg ochre dry mass/ml). On the other hand, the evaluation of

the already ambiguous fluorescence signal of the Pseudalert®/Quanti-Tray®/2000 method was interfered by autofluorescence of non-autoclaved ochre.

The highest recovery rate was achieved for *E. coli* by using the Colilert®-18/Quanti-Tray®/2000 method. In both ochre suspensions and well waters or deionised water a complete recovery of *E. coli* was detected. The spread plate technique using Lactose TTC Agar with Tergitol® 7 however yielded a mean recovery rate of 76 % for *E. coli* in two different ochre samples. The Colilert®-18/Quanti-Tray®/2000 when used for the quantification of *K. pneumoniae* and the spread plate method using Chromocult® Enterococci-Agar to quantify *E. faecalis* resulted in mean recovery rates of about 50-60 % to a complete recovery at most. Lower mean recovery rates of 16 %, 18 % and 14 % in ochre suspensions were obtained for the spread plate methods using Pseudomonas CN Agar, ampicillin dextrin agar or Legionella selective medium GVPC agar plates for the detection of *P. aeruginosa*, *A. hydrophila* and *L. pneumophila*, respectively. Recovery rates for the cultivation methods applied in the subsequent microcosm and column experiments are given in Table 21.

Table 21: Recovery rates of cultivation techniques for the quantification of target organisms in ochre suspension and well water. Given are the mean recovery rates for all day 0 microcosm samples and the maximal recovery rate found within these samples.

			Recovery rate [%]	
Cultivation method	**Target species**	**Sample type**	**Mean**	**Maximum**
Colilert®-18/Quanti-Tray®/2000	*E. coli*	Ochre suspension	Complete recovery	
	E. coli	Water	Complete recovery	
Colilert®-18/Quanti-Tray®/2000	*K. pneumoniae*	Ochre suspension	52	Complete recovery
	K. pneumoniae	Water	62	Complete recovery
Spread plate method using				
Chromocult® Enterococci-Agar	*E. faecalis*	Ochre suspension	64	Complete recovery
	E. faecalis	Water	42	68
Pseudomonas CN agar	*P. aeruginosa*	Ochre suspension	16	57
	P. aeruginosa	Water	25	58
Ampicillin-dextrin agar	*A. hydrophila*	Ochre suspension	18	44
	A. hydrophila	Water	17	34
GVPC agar	*L. pneumophila*	Ochre suspension	14	99
	L. pneumophila	Water	12	37

3.3 Microbiological examination of native ochre and well water samples

The ochre and well water samples from both dewatering and drinking water wells were analysed using microbiological methods to test for hygienically relevant bacteria and to characterise the samples microbiologically. The total cell count and heterotrophic plate count (HPC) of the water samples ranged from $2.5 \times 10^3 - 1.1 \times 10^6$ cells/ml and $1.5 \times 10^1 - 1.2 \times 10^5$ CFU/ml for the dewatering wells and was in the rage of 3×10^4 cells/ml and 6×10^1 CFU/ml for the drinking water wells. The HPC of the ochre samples ranged between $2.0 \times 10^7 - 2.2 \times 10^8$ CFU/g dry mass and $3.0 \times 10^6 - 2.1 \times 10^8$ CFU/g dry mass for the dewatering wells and drinking water wells, respectively.

Hygienically relevant bacteria were detected in seven well water samples and in three ochre samples. All samples, except from W. Tw-21, were from dewatering wells. Results are given in Table 22. Coliform bacteria were detected in three ochre and three water samples. *E. coli* and enterococci, the faecal indicator bacteria, were detected in one of these water samples, too. In one of the ochre samples, on the other hand, *Aeromonas* spp. were also found, as well as in six water samples, one from a drinking water well.

Table 22: Hygienically relevant bacteria detected in well water and ochre samples. All samples except from W. Tw-21 are from dewatering wells in opencast mines. *Duplicates each with a negative and a positive result.

	Coliform bacteria	***E. coli***	**Enterococci**	***Aeromonas* spp.**
Water samples	[MPN/100 ml]	[MPN/100 ml]	[CFU/100 ml]	[CFU/100 ml]
W. HR 904	< 1	< 1	0	1.0×10^1
W. HR 903	4.6×10^2	< 1; 1*	1.5	2.4×10^2
W. HR 838	< 1; 2*	< 1	0	0
W. HR 1172	< 1	< 1	0	1.5
W. HR 1173	< 1; 1*	< 1	0	1.5
W. W 5465	< 1	< 1	0	1.5
W. Tw-21	< 1	< 1	0	2
Ochre samples	[MPN/g dry mass]	[MPN/g dry mass]	[CFU/g dry mass]	[CFU/g dry mass]
O. VK 24	4.4×10^1	< 1	0	0
O. W 5465	4.1×10^1	< 1	0	6.9×10^2
O. 4616	9.5×10^1	< 1	0	0

The following coliform bacteria were identified in the samples by means of biochemical reactions (API 20 E®, bioMérieux):

- *Citrobacter koseri/amalonaticus* (excellent identification) in W. HR 903
- *Escherichia coli* (excellent identification) in W. HR 903
- *Enterobacter cloacae* (good identification) in W. HR 1173
- *Serratia* spp. (very good identification at the genus level) in O. VK 24
- *Enterobacter cloacae* (good identification) in O. W 5465
- *Serratia* spp. (good identification at the genus level) in O. W 5465

Besides, the biochemical identification by means of API 20 NE® (bioMérieux) confirmed the finding of *Aeromonas* spp. in all water samples (excellent identification).

3.4 Physico-chemical characterisation of ochre

The ochre samples which were used for the microcosm, column and disinfection experiments were chemically characterised by the determination of selected elements by inductively coupled plasma optical emission spectrometry (ICP-OES) according to DIN EN ISO 11885 (measurements performed by IWW Zentrum Wasser). Results are given in Table 23 along with results of the determination of dry residue and ignition loss of the ochre samples, as well as pH values of suspensions of the ochre samples in deionised water (0.11 g wet mass/ml).

The results of the elemental analysis show, as expected, that iron is the dominating element in all ochre samples but also that the chemical composition of the different ochre samples still varies more or less. The iron content ranges from 268 g/kg dry mass in ochre sample O. HS 1362 to 547 g/kg dry mass in ochre sample O. W 5465. The ochre samples from the drinking water wells in Berlin contain less iron (380 – 462 g/kg dry mass) than the samples from the dewatering wells (477 – 547 g/kg dry mass), except from sample O. HS 1362. On the other hand, the samples from the drinking water wells are characterised by containing considerably higher amounts of calcium than the other ochre samples (49.9 – 65.4 g/kg dry mass vs. 3.2 – 21.1 g/kg dry mass) and also a bit more manganese (1.15 – 2.52 g/kg dry mass vs. 0.0291 – 1.13 g/kg dry mass). Ochre sample O. HS 1362, in comparison, contains a remarkably high amount of aluminium (7.1 g/kg dry mass vs. 0.027 – 1.69 g/kg dry mass) and copper (3.98 g/kg dry mass vs. < 0.003 – 0.259 g/kg dry mass in the other samples, except O. Tw-22 which contains 1.5 g/kg dry mass).

The loss on ignition, as an estimate of organic carbon content, is quite similar for the different ochre samples, ranging from 11.3 % to 15.6 %, with the exception of sample O. VK 24 which yielded an ignition loss of 21.8 %. The pH of most of the ochre suspensions is around neutral (pH 6.5 – 7.7), only those of the samples from the opencast mine Reichwalde and Nochten in northeast Saxony O. NW 83 and O. VK 24 are acidic with pH values of 5.7 and 4.6, respectively.

These results reveal that hygienically relevant bacteria, introduced into ochre incrusted wells or spiked into ochre samples from different wells, as in the case of the microcosm and column experiments in the present study, are confronted with different ochre compositions and might therefore also experience different stress factors or nutrient conditions.

Table 23: Physio-chemical characterisation of ochre samples used for microcosm, column and disinfection experiments. Elements in g/kg dry residue. Compounds in %, calculated from the amount of the element. Dry residue (D.R.) and ignition loss (I.L.) in %. pH of an suspension of the respective ochre in deionised water (0.11 g wet mass/ml). - = not determined. **Minimum / maximum values** of some parameters.

	2011				2012			2013			
Ochre	VK 24	WR 2378	HS 1362	BWB	H 1424	W 5465	NW 83	Tw-21	To-05	FRI-25	Tw-22
Fe	477	510	**268**	380	518	**547**	530	395	416	417	462
FeOOH	75.9	81.1	42.6	60.5	82.4	87.0	84.3	62.8	66.2	66.3	73.5
P	-	-	-	-	-	5.9	14.2	83.6	107	92.4	75.1
PO_4	-	-	-	-	-	1.8	4.4	25.6	32.8	28.3	23.0
Ca	< 4	< 6	10.1	49.9	11.8	21.1	**3.2**	61.2	**65.4**	62	54.7
$CaCO_3$	-	-	2.5	12.5	2.9	5.3	0.8	15.3	16.3	15.5	13.7
Al	1.08	1.08	**7.1**	0.196	**0.027**	0.61	1.69	0.11	0.037	0.18	0.075
$Al(OH)_3$	0.31	0.31	2.1	0.06	-	-	-	-	-	-	-
Cu	0.135	0.259	**3.98**	0.148	< 0.003	0.12	< 0.003	0.029	**0.015**	0.036	1.5
$CuCO_3$	0.03	0.05	0.77	0.03	-	-	-	-	-	-	-
Mn	**0.029**	1.13	0.862	**2.52**	0.33	0.44	0.23	1.15	2.1	1.99	1.57
MnO_2	0.005	0.18	0.14	0.4	0.05	-	-	-	-	-	-
Mg	< 4	< 6	< 7	< 6	< 0.6	< 0.6	< 0.6	1.35	1.45	1.7	1.2
Na	< 4	< 6	< 7	< 6	< 0.6	< 0.6	< 0.6	1.12	1.6	1.2	< 0.7
K	< 4	< 6	< 7	< 6	< 0.6	< 0.6	< 0.6	< 0.6	0.63	< 0.8	< 0.7
Si	< 4	< 6	< 7	< 6	**2.89**	3.02	3.23	4.73	4.36	**7.3**	5.77
SiO_2	-	-	-	-	0.62	0.65	0.69	-	-	-	-
Ni	0.075	0.194	**0.293**	0.046	0.061	0.061	0.052	0.027	0.037	0.040	**0.026**
As	-	-	-	-	-	0.013	0.097	-	-	-	-
Pb	< 0.02	< 0.03	0.065	< 0.03	0.008	< 0.003	< 0.3	< 0.003	< 0.003	< 0.003	< 0.003
Cd	< 0.002	< 0.003	< 0.003	< 0.003	< 0.003	< 0.003	< 0.003	0.002	0.001	0.001	0.002
Cr	< 0.008	0.033	0.173	< 0.02	0.003	< 0.003	< 0.003	0.003	0.002	0.002	0.003
Zn	< 0.08	< 0.2	0.138	0.119	0.023	< 0.1	< 0.1	0.047	0.024	0.17	0.069
D.R.	46.7	**49.9**	27.8	20.4	29.6	21.9	40.6	22.9	37.6	35.0	**18.3**
I.L.	**21.8**	**11.3**	**11.3**	14.6	13.1	11.9	14.1	15.6	13.1	13.3	14.9
pH	**4.6**	6.5	6.9	7.6	6.6	7.6	5.7	7.4	**7.7**	6.8	7.0

Scanning electron microscopic examination of ochre samples revealed a particulate structure of the samples, which could be quite heterogeneous in its composition. It was not possible to clearly differentiate ochre samples from different wells from one another. Bacteria attached to the ochre could also not clearly be detected, which, however, might be due the sample preparation, especially the drying of the samples, and the difficulties in the scanning electron microscopic examination of ochre itself. It was not easy to gain a sharp image and often the sample appeared to be shining. Examples of scanning electron microscopic pictures of three different ochre samples are shown in Figure 17.

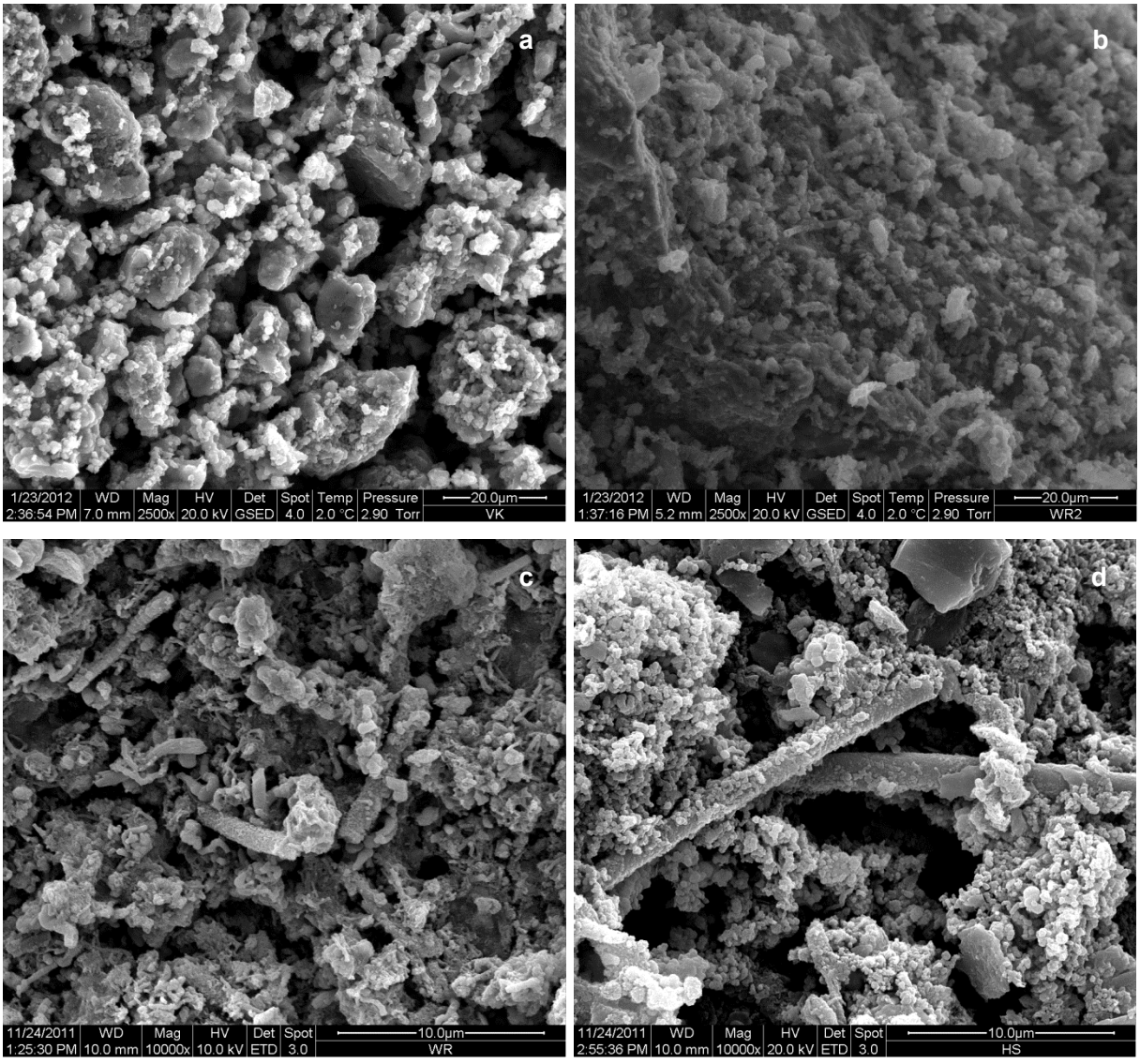

Figure 17: Scanning electron micrographs of ochre samples. (a) O. VK 24 (2500x), (b) O. WR 2378 (2500x), (c) O. WR 2378 (10,000x), (d) O. HS 1362 (10,000x). The particulate structure of the ochre samples could be quite heterogeneous in its composition. Bacteria were not clearly visible.

3.5 Microcosm experiments – survival of hygienically relevant bacteria in suspensions of ochre samples and in well waters in a batch system

As described above (cf. page 52), the strategy of the present study was to simulate the contamination of an ochre-incrusted well by hygienically relevant bacteria to elucidate if such incrustations can act as sink and source for pathogens. For that purpose, indicator bacteria of faecal and non-faecal contamination and opportunistic pathogens were spiked into suspensions of ochre samples from different wells, followed by the determination of their concentration over time, to first check the potential of those bacteria to survive in an ochre containing matrix.

In microcosm experiments, in 100 ml Erlenmeyer flasks, the survival of the different target organisms over time was tested in suspensions of different ochre samples and in the later experiments also, for comparison, in well water samples from the respective well.

Since both the ochre and well water were applied as native samples and were not sterilised before using it for the microcosm experiments, the heterotrophic plate count (HPC) was determined at the beginning and at the end of the experiments. The HPC serves as a measure of the amount of culturable autochthonous bacteria and fungi present in a sample.

Furthermore, the pH of the ochre suspensions and well water samples was measured also at the beginning and at the end of the experiments.

3.5.1 Planktonic vs. ochre associated target bacteria

In the initial procedure of the first microcosm experiments, in which the survival of *E. coli* or *K. pneumoniae* in ochre suspension over time was studied, the water phase and solid matter content of the suspension were separated by centrifugation (5000 x g, 10 min, 4 °C) to differentiate between the amounts of planktonic cells versus cells associated with ochre. Quantification of the spiked bacteria in the whole ochre suspension and in the supernatant by cultivation revealed that only a minor portion of target organisms was present in the water phase. On average only about 10 % of *E. coli* and 0.3 % of *K. pneumoniae* were detected in the water phase (Supernatant 1 Figure 18) of the ochre suspension. Resuspending the ochre sediment with deionised water and washing it after a second centrifugation step with a dispersing solution transferred only few target organisms from the solid phase into the respective water phase (Supernatant 2 and 3 Figure 18). It added up to about 1 % or 0.1 % on average of the total number of *E. coli* or *K. pneumoniae* present in the whole ochre suspension. The major part of target bacteria, however, was detected in the remaining sediment (Figure 18).

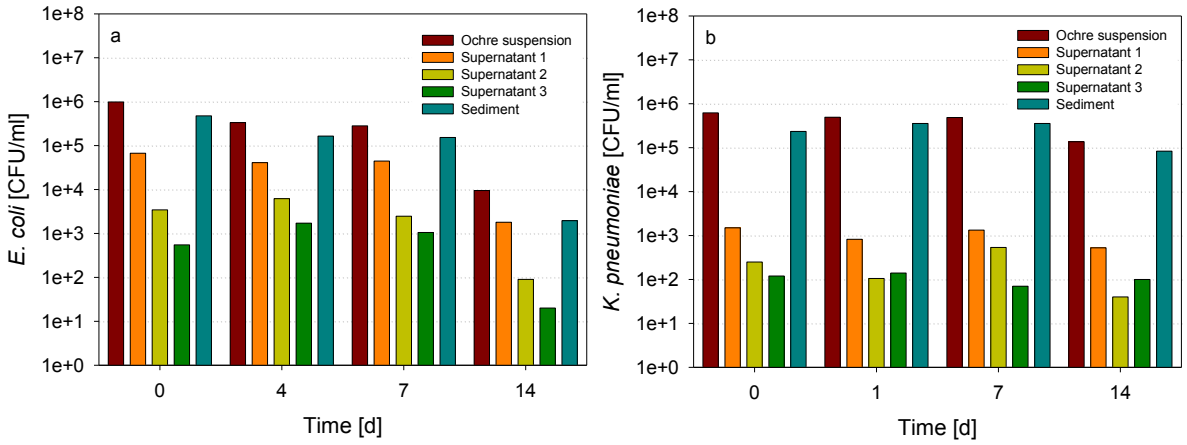

Figure 18: Concentration of planktonic vs. with ochre associated *E. coli* or *K. pneumoniae* over time. Ochre suspension: Suspensions spiked with the bacteria; Supernatant: Liquid phase of the ochre suspension (1) after centrifugation (5000 x g, 10 min), (2) after resuspension with deionised water and centrifugation, (3) after washing the sediment with a dispersing solution (45 min, shaker) and centrifugation; Sediment: remaining solid phase of the initial ochre suspension after decanting Supernatant 1-3.

Since the target bacteria were mainly associated with the sediment after centrifugation, and the ochre particles did not interfere with the detection of the respective organism by cultivation, the centrifugation and washing steps were omitted in the subsequent microcosm experiments.

3.5.2 Microcosm experiments with suspensions of four different ochre samples

As it was obvious from the results of the physico-chemical characterisation of ochre samples (cf. section 3.4) that "the typical ochre" does not exist, but that the composition of the ochreous incrustations from wells of different locations can be quite diverse, it was studied if the survival of the target bacteria in suspensions of such different ochre samples also differs from one another.

These microcosm experiments, comparing the survival of the six target bacteria in four different ochre samples, showed that the rate of survival in a culturable state over time can be different for the diverse bacteria in the respective samples (Figure 19). In the case of these four samples, this mainly applies for *E. faecalis*, *P. aeruginosa* and *L. pneumophila*.

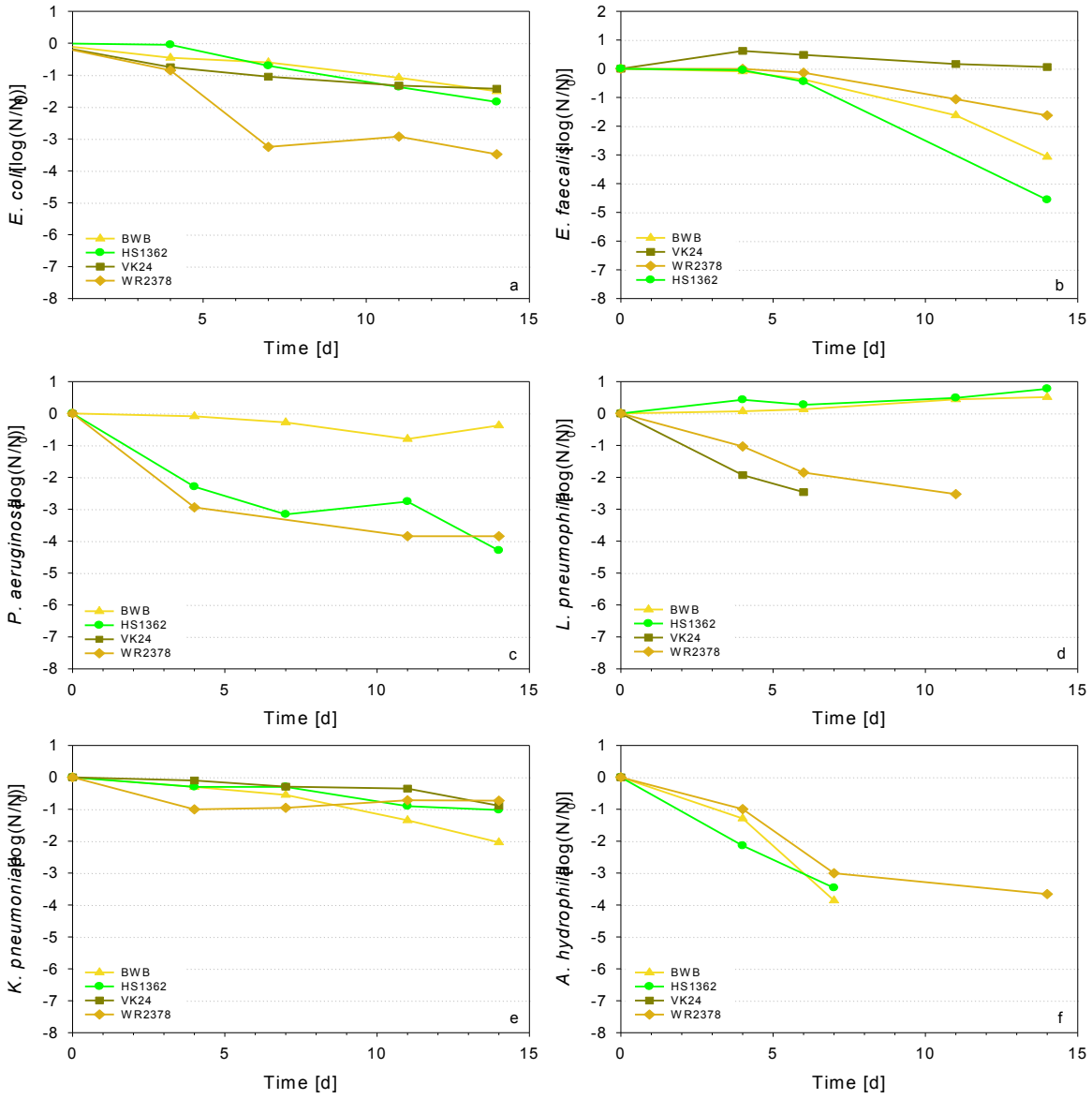

Figure 19: Survival of target organisms over time in suspensions of ochre samples from different wells and locations. Samples were spiked with 1 x 10^6 cells/ml. N: Concentration [MPN or CFU/ml] at the respective sampling day; N_0: Concentration [MPN or CFU/ml] at day 0.

E. faecalis showed the most pronounced variation in the number of colony forming units over time in the different ochre samples tested. In ochre sample VK 24 the number of culturable *E. faecalis* was nearly constant; in ochre samples WR 2378, BWB and HS 1362 it decreased during 14 days about two, three and five orders of magnitude. *L. pneumophila* survived in ochre BWB and HS 1362 without a decrease in the number of colony forming units throughout the experiment. The numbers even slightly increased from about 1.5 x 10^4 CFU/ml at day 0 up to about 5.0 x 10^4 CFU/ml and 9.0 x 10^4 CFU/ml at day 14, respectively. While in ochre samples VK 24 and WR 2378 *L. pneumophila* was no longer detectable by cultivation after day 7 and after day 11. *P. aeruginosa* showed only a slight decrease in numbers of colo-

ny forming units, of less than half an order of magnitude, in ochre BWB after 14 days, whereas in ochre samples HS 1362 and WR 2378 it dropped about four log units. In ochre VK 24 *P. aeruginosa* was detected only on day 4, day 6 and day 11 at the detection limit of 5 CFU/ml.

K. pneumoniae, *E. coli* and *A. hydrophila*, however, showed less variation in numbers of colony forming units over time in the different ochre samples tested. For *K. pneumoniae* a decrease in colony forming units of about one order of magnitude over the period of 14 days was detected in ochre samples HS 1362, VK 24 and WR 2378; only in sample BWB a decrease of about two log units was observed. The number of culturable *E. coli* declined in ochre samples BWB, HS 1362 and VK 24 about 1.5 and in ochre WR 2378 3.5 orders of magnitude. *A. hydrophila*, on the other hand, showed a sharp decline in numbers of colony forming units in all ochre samples; being no longer detectable in ochre VK 24 after day 0 and in ochre samples BWB and HS 1362 after day 7. In ochre WR 2378 the number of colony forming units of *A. hydrophila* decreased 3.7 log units over the period of 14 days.

Interestingly not all target organisms showed the same response to the exposure to the respective ochre sample. A pronounced difference could be seen especially for ochre VK 24. *E. faecalis*, *K. pneumoniae* and *E. coli* showed no or only a slight decrease in numbers of colony forming units over the course of 14 days in this ochre sample, whereas *P. aeruginosa, A. hydrophila* and *L. pneumophila* reacted with a fast loss of culturability to the exposure to this ochre. Furthermore, *P. aeruginosa* and *L. pneumophila* survived without a decline in numbers of colony forming units in ochre BWB, while *E. faecalis* and *A. hydrophila* showed a significant decrease in numbers of colony forming units in this sample. The same is true for ochre HS 1362 and *L. pneumophila* versus *A. hydrophila*, *P. aeruginosa* and *E. faecalis*. The first bacterium showed no reduction in numbers of colony forming units in this ochre, while the others respond to it with a pronounced drop of culturability.

The HPC measurements of the ochre suspensions at the beginning and at the end of the experiments yielded the data given in Figure 20. At day 0 HPC values of $3.0 \times 10^5 - 1.3 \times 10^6$ CFU/ml ochre suspension were detected. For normalizing the amount of ochre dry mass contained in the respective sample, these values were converted to $1.4 \times 10^7 - 3.0 \times 10^7$ CFU/g ochre dry mass. Over the course of the experiments the HPC values of the four ochre suspensions increased 0.8 log units on average.

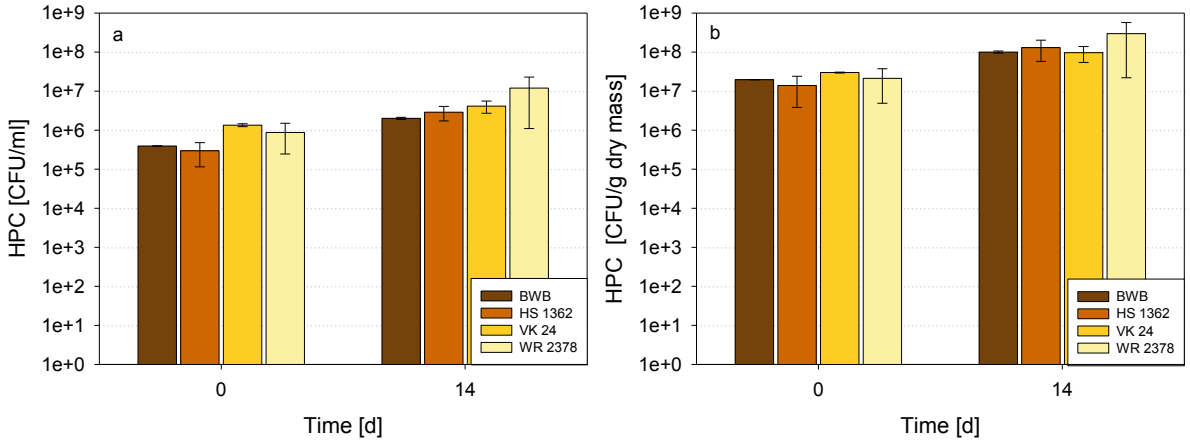

Figure 20: Heterotrphic plate count (HPC) of suspensions of ochre samples from different wells and locations.

These data show that the ochre samples all contain a considerable content of native microflora, in particular taking into consideration that the culturable part of it is normally only a small fraction of the overall population. The slight increase of the HPC values over the course of the experiments, might indicate a growth of heterotrophic microorganisms over time, but could also be based just on an increase in culturability of these autochthonous bacteria and fungi. However, it clearly shows that the hygienically relevant bacteria, spiked into the ochre suspensions, have to cope with the native microbiota if to survive.

The pH-value was constant over the course of the experiments. The mean pH values of the ochre suspensions were 7.5, 7.0, 4.7 and 6.8 for ochre samples BWB, HS 1362, VK 24 and WR 2378, respectively.

3.5.3 Microcosms experiments with ochre suspensions and the respective well water

In order to get a general idea of the survival potential of the different hygienically relevant bacteria in the oligotrophic environment of a water well, and to assess the effect of ochre on the survival of these bacteria, water from the wells ochre samples were taken from was used as another microcosm medium. The results of these experiments are displayed in the graphs in the next two sections; in the first section for samples from three different dewatering wells and in the second for samples from three drinking water wells. The figures show mean values of two consecutive experimental runs; standard deviations are given in the tables in annex 6.2.

The results of the microbiological characterisation of the ochre suspensions and well water samples and the pH-values of the samples are given afterwards (page 80ff.).

3.5.3.1 Survival of hygienically relevant bacteria over time in ochre suspensions and water samples from dewatering wells

In ochre suspensions from dewatering wells, *E. faecalis* showed the smallest decline in numbers of colony forming units over time of the six target organisms tested, followed by *L. pneumophila*. For *E. faecalis* only 0.1 to 0.4 log units decrease was detected. In ochre sample W 5465 the numbers of culturable *E. faecalis* even slightly increased over time, but did not exceed the number of cells added at the beginning (Figure 21 b). *L. pneumophila* showed a decline of 0.3 to 1.2 log units in the ochre suspensions over the course of 14 days (Figure 22 b).

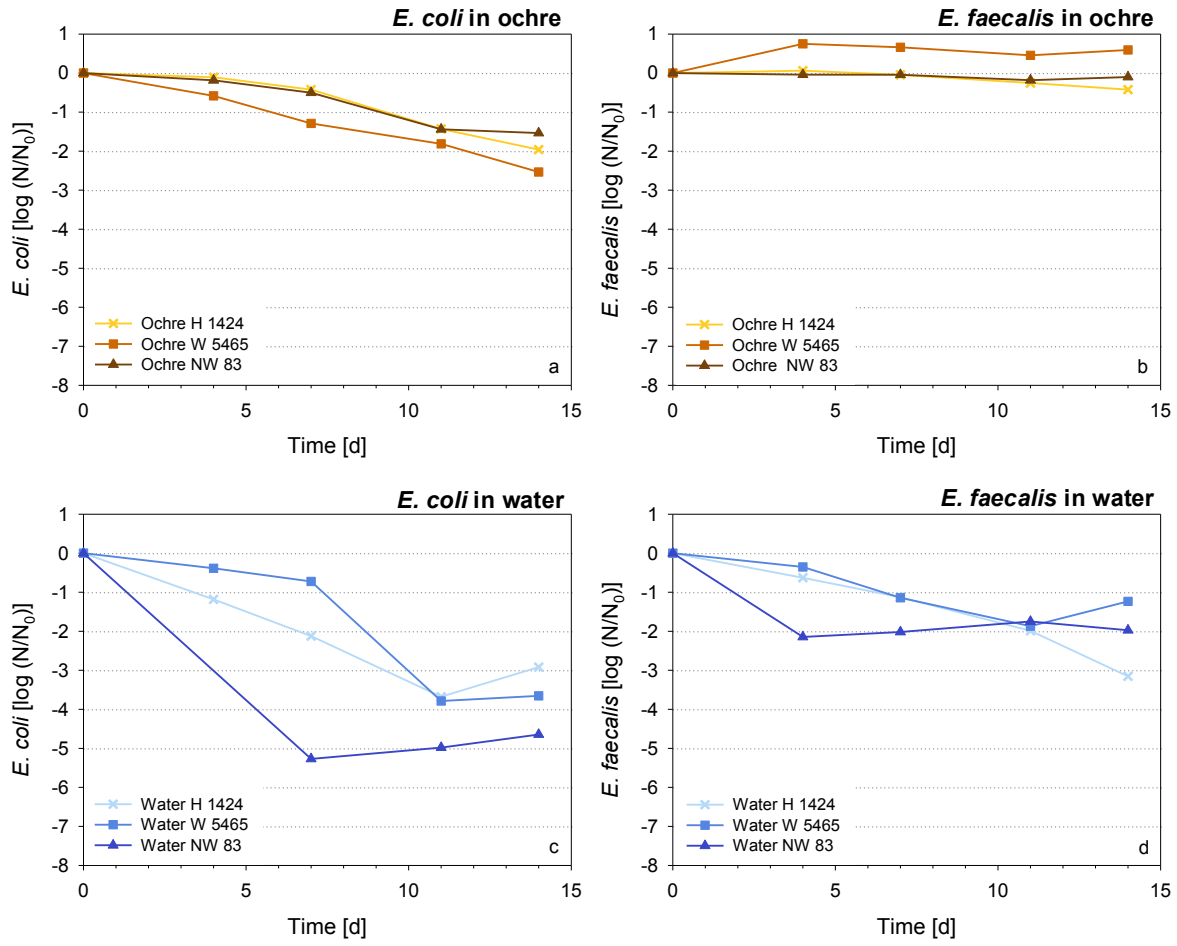

Figure 21: Survival of *E. coli* (a, c) and *E. faecalis* (b, d) in suspensions of ochre samples and in water samples from dewatering wells, respectively. Mean values of two consecutive runs of microcosm experiments. Standard deviations are given in Table 33. Samples were spiked with 1 x 10^6 cells/ml (H 1424) or with 1 x 10^8 cells/ml. *E. coli* in Water H 1424 at day 4 and in Water W 5465 at day 7 only detected in run I or run II, respectively. *E. coli* not detected at day 4 in Water NW 83. N: Concentration [MPN or CFU/ml] at the respective sampling day; N_0: Concentration [MPN or CFU/ml] at day 0.

A similar decrease of about 2 log units on average after 14 days in suspensions of ochre samples from dewatering wells was detected for *E. coli*, *K. pneumoniae, P. aeruginosa*, and

A. hydrophila (Figure 21 a, Figure 23 a, Figure 22 a and Figure 23 b). Though, the variation in the decline of colony forming units over time in the different ochre samples was stronger for *P. aeruginosa* and *A. hydrophila* than for the other two. Numbers of culturable *P. aeruginosa* dropped 3.2 log units in ochre NW 83, whereas in ochre H 1424 only a decrease of 0.5 log units was detected. Similarly, numbers of culturable *A. hydrophila* showed a more significant decline in the suspension of ochre NW 83 than in the other two samples, 3.1 log units vs. 1.5 and 1.7 log units over 14 days.

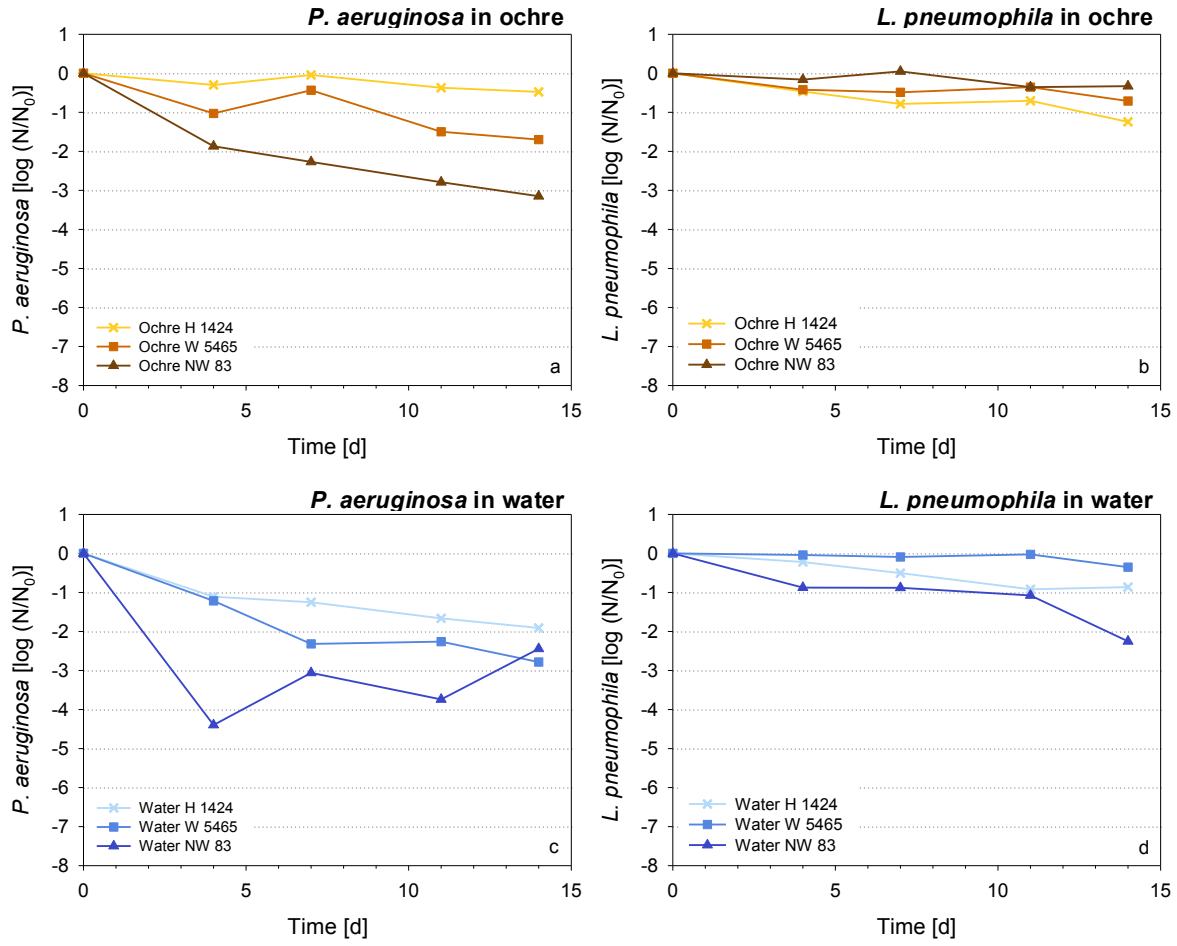

Figure 22: Survival of *P. aeruginosa* (a, c) and *L. pneumophila* (b, d) in suspensions of ochre samples and in water samples from dewatering wells, respectively. Mean values of two consecutive runs of microcosm experiments. Standard deviations are given in Table 34. Samples were spiked with 1 x 10^6 cells/ml (H 1424) or with 1 x 10^8 cells/ml. *P. aeruginosa* and *L. pneumophila* in Water NW 83 at day 4 and 14, and at day 11 and 14, respectively, were only detected in run II. N: Concentration [MPN or CFU/ml] at the respective sampling day; N_0: Concentration [MPN or CFU/ml] at day 0.

For all target organisms, the decrease in numbers of colony forming units over time was on average higher in the well water samples than in the ochre suspensions. The most pronounced decrease of 4.1 and 3.7 log units on average was detected for *K. pneumoniae* and *E. coli*, respectively. However, both species showed a marked decline (5.0 and 4.7 log units, respective-

ly) especially in well water sample NW 83 (Figure 23 c, Figure 21 c), which was characterised by a rather low pH of 3.4 (see Table 24).

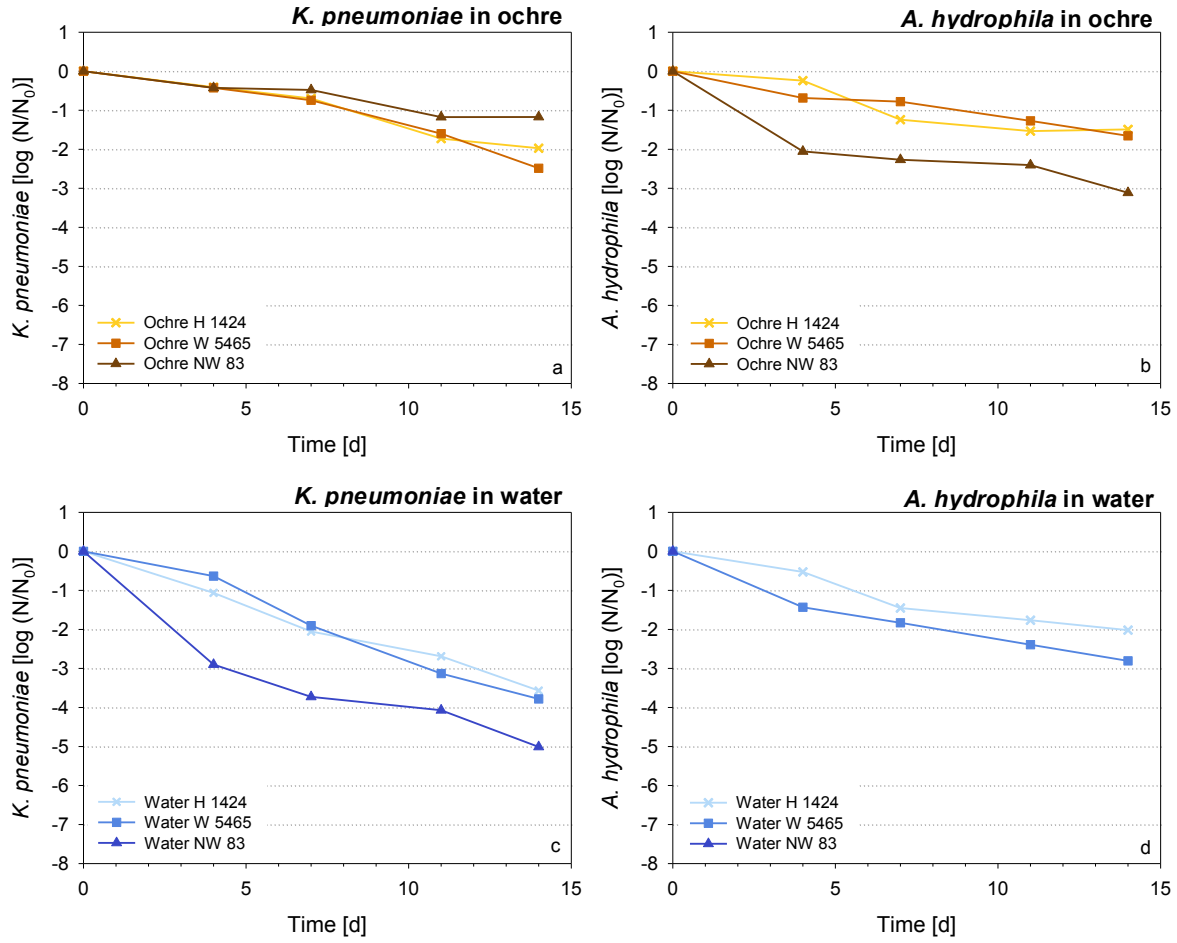

Figure 23: Survival of *K. pneumoniae* (a, c) and *A. hydrophila* (b, d) in suspensions of ochre samples and in water samples from dewatering wells, respectively. Mean values of two consecutive runs of microcosm experiments. Standard deviations are given in Table 35. Samples were spiked with 1 x 10^6 cells/ml (H 1424) or with 1 x 10^8 cells/ml. *K.* pneumoniae in Ochre W 5465 at day 14 was only detected in run II. *A. hydrophila* in Water NW 83 was detected in run I at day 0 and in run II at all days but day 0. N: Concentration [MPN or CFU/ml] at the respective sampling day; N_0: Concentration [MPN or CFU/ml] at day 0.

A. hydrophila, likewise, was not detected by cultivation in water NW 83 at every sampling day of the two consecutive experimental runs. In the first run *A. hydrophila* was only detected by cultivation on day 0 and in the second run on days 4 to 14, but not on day 0. Therefore, the chart (Figure 23 d) displaying the ratio of colony forming units at a particular sampling day to the number of colony forming units at day 0 [$\log(N/N_0)$], does not show a graph for *A. hydrophila* in water NW 83. *P. aeruginosa* and *E. faecalis* (Figure 22 c and Figure 21 d), similarly, showed a sharp decline in numbers of colony forming units in water NW 83 in the beginning of the experiments. Also, the mean decrease of numbers of culturable

A. hydrophila, *P. aeruginosa* and *E. faecalis* in all water samples at day 14 was quite similar; 2.4 log units for the first two species and 2.1 log units for the last.

The least decline in colony forming units in the water samples over time was detected for *L. pneumophila* (Figure 22 d). At day 14, it showed a decline of 0.4 and 0.9 log units in water samples W 5465 and H 1424, respectively. Only in water sample NW 83 a decrease of 2.3 log units was observed on day 14.

3.5.3.2 *Survival of hygienically relevant bacteria over time in ochre suspensions and water samples from drinking water wells*

In suspensions of ochre samples from drinking water wells, *E. coli* demonstrated the most pronounced decrease of colony forming units of the six target organisms tested (cf. Figure 24, Figure 25 and Figure 26).

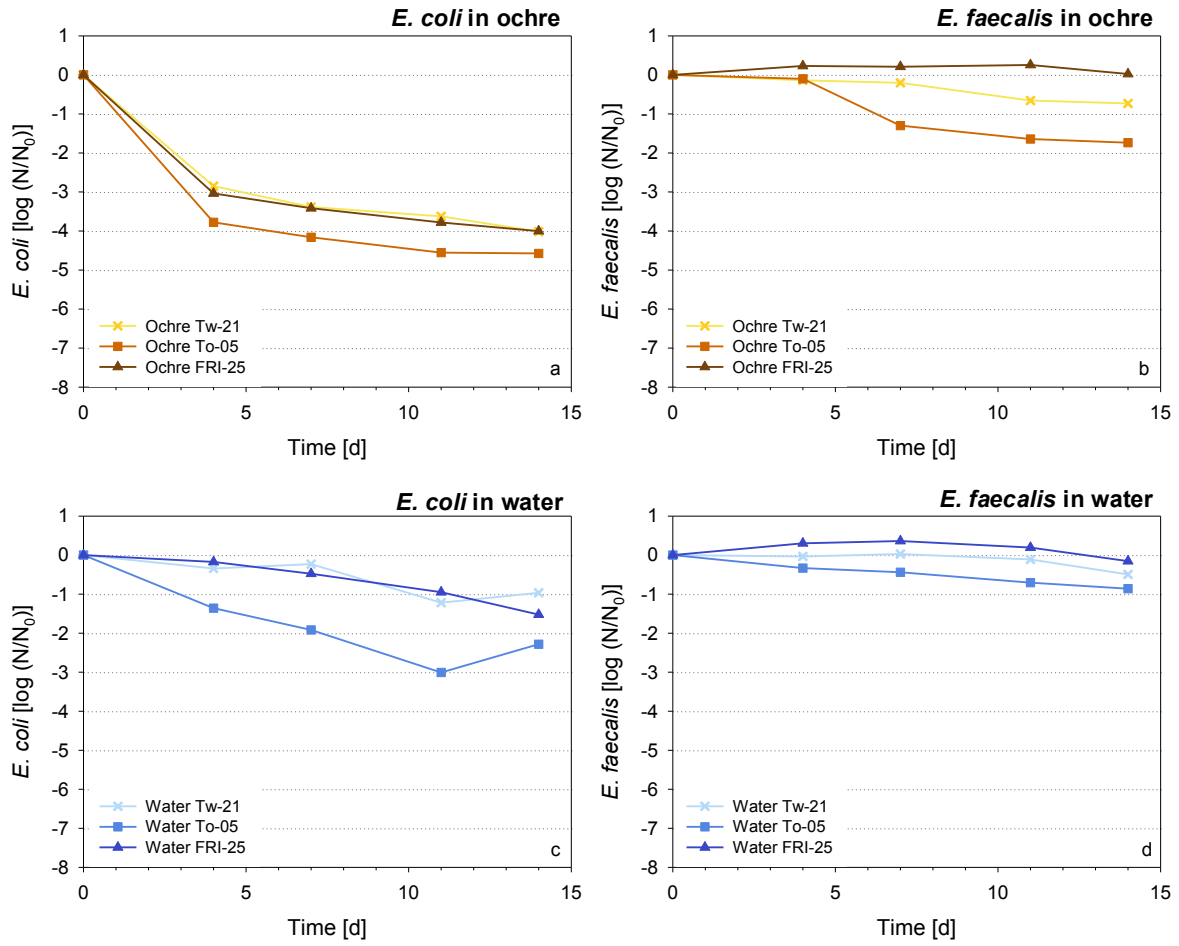

Figure 24: Survival of *E. coli*, (a, c) and *E. faecalis* (b, d) in suspensions of ochre samples and in water samples from drinking water wells, respectively. Mean values of two consecutive runs of microcosm experiments. Standard deviations are given in Table 36. Samples were spiked with 1 x 10^8 cells/ml. For ochre Tw-21 sampling day 4 and 11 was only tested in run I. N: Concentration [MPN or CFU/ml] at the respective sampling day; N_0: Concentration [MPN or CFU/ml] at day 0.

Over the course of the experiments numbers of culturable *E. coli* dropped 4.0 log units in ochre samples Tw-21 and FRI-25, and 4.6 log units in ochre sample To-05 (Figure 24 a). For the other target organisms (Figure 24 b, Figure 25 and Figure 26), however, a decrease of about 1 to 2 log units was detected with the exception of *E. faecalis* in ochre suspension FRI-25, in which it showed no decline at all over the course of 14 days (Figure 24 b).

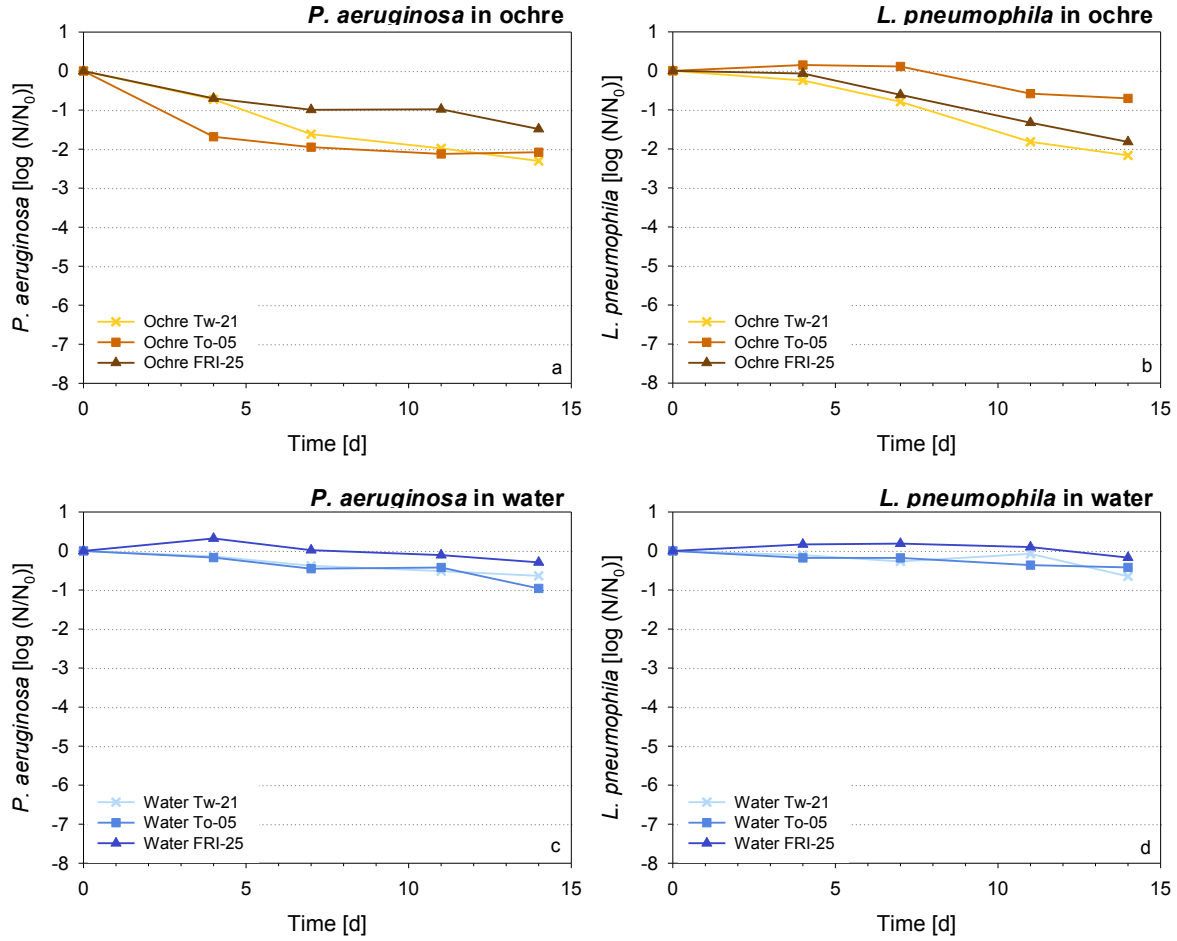

Figure 25: Survival of *P. aeruginosa* (a, c) and *L. pneumophila* (b, d) in suspensions of ochre samples and in water samples from drinking water wells, respectively. Mean values of two consecutive runs of microcosm experiments. Standard deviations are given in **Table** 37. Samples were spiked with 1 x 10^8 cells/ml. For ochre Tw-21 sampling day 4 and 11 was only tested in run I. N: Concentration [MPN or CFU/ml] at the respective sampling day; N_0: Concentration [MPN or CFU/ml] at day 0.

On average, no significant difference in numbers of colony forming units over time, detected in water samples versus ochre suspensions, was observed for *E. faecalis*, *K. pneumoniae* and *A. hydrophila* (Figure 24 b, d and Figure 26). Whereas *E. coli*, *P. aeruginosa* and *L. pneumophila* displayed a minor decrease in numbers of colony forming units over time in water samples than in ochre suspensions (Figure 24 a, b and Figure 25). On average, a decrease of 1.6 log units in water samples vs. 4.2 log units in ochre samples was observed for *E. coli*. Similar-

ly, *P. aeruginosa* and *L. pneumophila* showed a mean log reduction of 0.6 and 0.4, respectively, in water samples compared to a log reduction of 2 and 1.6 in ochre samples.

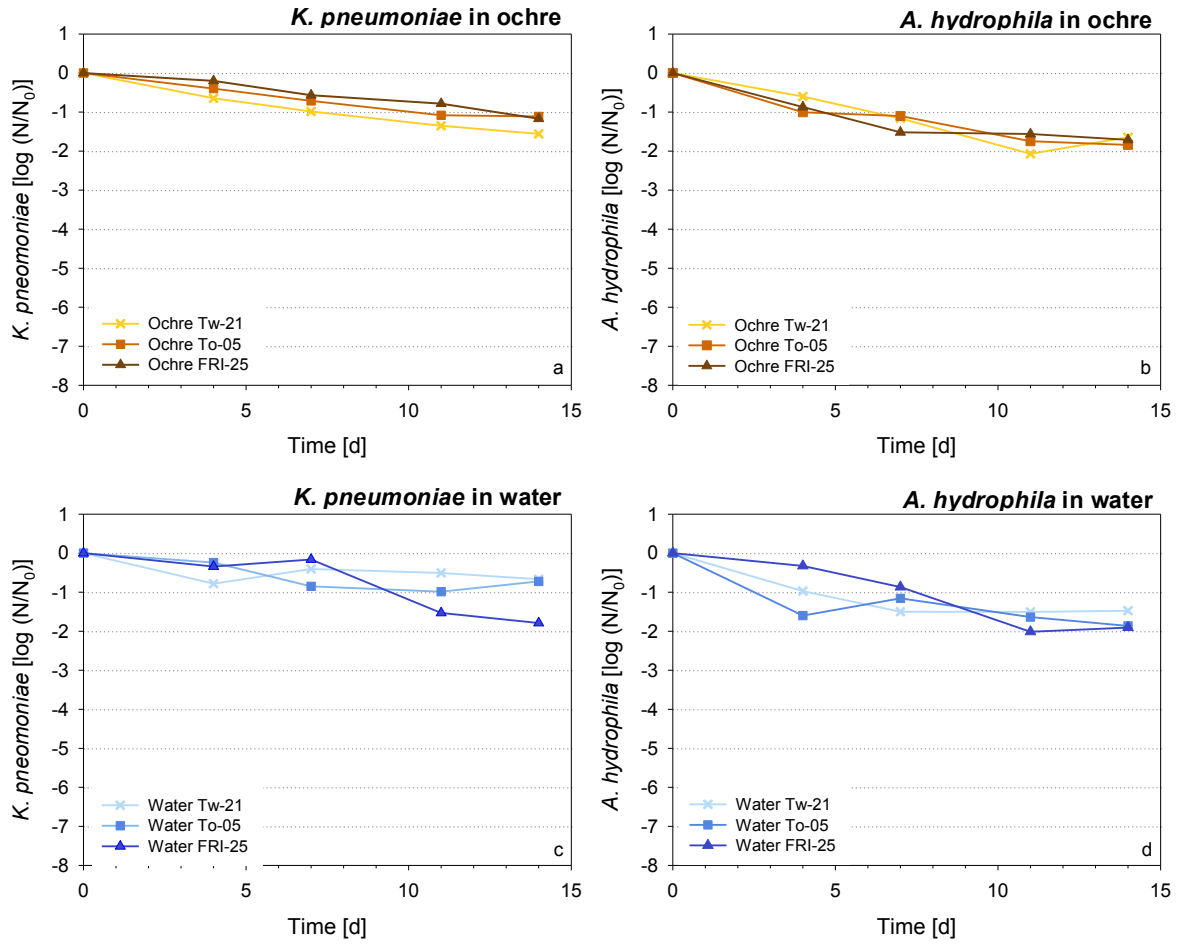

Figure 26: Survival of *K. pneumoniae* (a, c) and *A. hydrophila* (b, d) in suspensions of ochre samples and in water samples from drinking water wells, respectively. Mean values of two consecutive runs of microcosm experiments. Standard deviations are given in **Table** 38. Samples were spiked with 1 x 10^8 cells/ml. For ochre Tw-21 sampling day 4 and 11 was only tested in run I. N: Concentration [MPN or CFU/ml] at the respective sampling day; N_0: Concentration [MPN or CFU/ml] at day 0.

From all microcosm experiments conducted, none of it resulted in an increase in numbers of target organisms over time compared to the number of cells added to the ochre suspensions at the beginning of the experiment. But a part of the population of each target organism survived 14 days in a culturable state in the majority of ochre samples tested. In all microcosm experiments, in which ochre suspensions and well water samples were used, in any sample a part of the population of each target organism survived 14 days in a culturable state.

3.5.3.3 Microbiological characterisation and pH of ochre suspensions and well waters

As already mentioned in sections 3.5 and 3.5.2, the heterotrophic plate count (HPC) method was applied to get an impression of the amount of indigenous microbiota present in the native samples used for the microcosm experiments. The HPC determined for the ochre and well water samples at the beginning and at the end of the experiments allows for a comparison of the dimension of heterotrophic bacteria and fungi the target organisms are confronted with in the different samples and the recognition of possible changes over the course of the experiment. The additional microscopic enumeration of all cells contained in the well water samples, the total cell count (TCC), which could not be achieved for the ochre suspensions because of the ochre particles interfering with the method, offers the opportunity to also quantify the non-culturable part of the native microflora. The results of the ochre and water samples from dewatering wells are given in Figure 27.

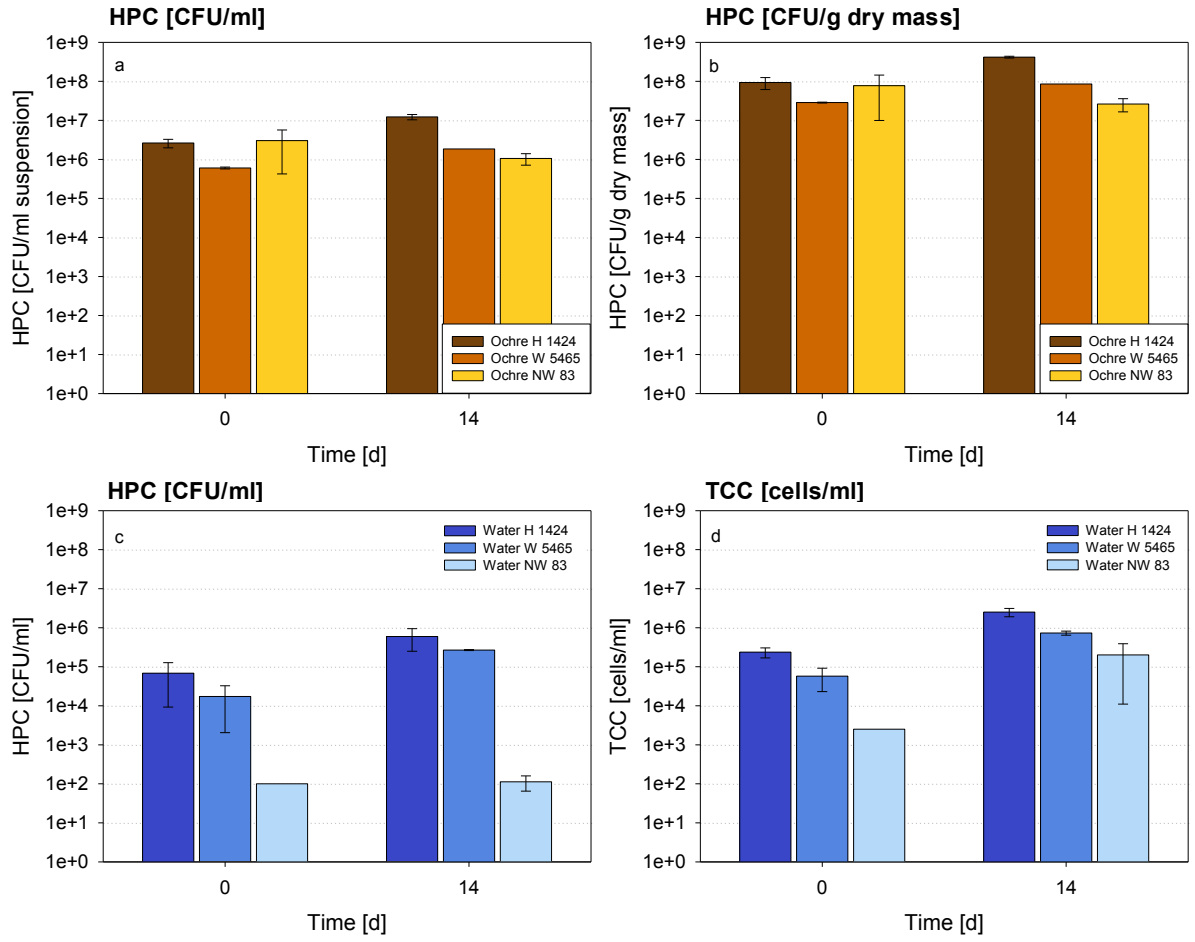

Figure 27: Heterotrphic plate count (HPC) of suspensions of ochre samples and of water samples from dewatering wells, as well as total cell count (TCC) of the water samples. Mean values with standard deviation of two consecutive runs of microcosm experiments. Values determined at the beginning (d0) and at the end (d14) of the runs. (a) HPC in CFU/ml ochre suspensions, as determined in the experiments; HPC of ochre W 5465 at d14 only determined in run II; (b) HPC in CFU/g ochre dry mass, calculated from the values illustrates in (a) and the amount of ochre dry mass contained in the suspensions; (c) HPC of the water samples; (d) TTC of the water samples; HPC and TCC of water sample NW 83 at day 0 only determined in run II.

At the beginning of the experiments the mean HPC of the ochre suspensions ranged from $6.0 \times 10^5 - 3.0 \times 10^6$ CFU/ml which corresponds to $2.9 \times 10^7 - 9.3 \times 10^7$ CFU/g ochre dry mass (Figure 27 a and b). During the experiments the HPC of the suspensions of ochre H 1424 and W 5465 increased about 0.7 and 0.5 log units, respectively. The HPC of ochre suspension NW 83 decreased by 0.2 log units. This shows that the magnitude of the native heterotrophic bacteria and fungi contained in different samples is quite similar and more or less constant over the course of the experiments.

The mean HPC of the water samples (Figure 27 c) H 1424 and W 5465, at the beginning of the experiments, was 6.8×10^4 CFU/ml and 1.7×10^4 CFU/ml, respectively. The HPC of water sample NW 83, however, was only 1.0×10^2 CFU/ml and was just determined in the second run of the experiment. Over the course of the experiments, the HPC of water samples H 1424 and W 5465 increased on average by 1.2 and 1.5 log units, respectively. Whereas the HPC of water sample NW 83 even slightly decreased by 0.2 log units during the second run of the experiment. The total cell count (TCC) in the water samples (see Figure 27 d) resulted in the following figures: On day 0, the mean TCC of water samples H 1424 and W 5465 was 2.4×10^5 cells/ml and 5.7×10^4 cells/ml, respectively. Whereas the TCC of the water sample NW 83 at day 0 could just be determined in the second experimental run and resulted in 2.5×10^3 cells/ml. The TCC increased during the experiments by about one log unit for water samples H 1424 and W 5465, and by 2.2 log units for sample NW 83 in the second experimental run. These results show that the slightly alkaline water samples (pH values are given in Table 24), H 1424 and W 5465, contain about one to two orders of magnitude less culturable heterotrophs per ml sample in the microcosms than the respective ochre suspensions. For the acidic water sample NW 83, however, the difference is even much more pronounced; it contains about four orders of magnitude less culturable HPC microbiota per ml than the respective ochre suspension. Over the course of the experiments, the amount of native microbiota in the water samples increased about one to two log units. But only for water samples H 1424 and W 5465 also the fraction of culturable heterotrophs increased while for water sample NW 83 this number was more or less constant. The minor fraction of culturable heterotrophs in water sample NW 83 is reflected by HPC to TCC ratios of 4 % at day 0 of the second run and a mean ratio of 0.7 % at day 14. Whereas this ratio was on average 24 % and 22 % at day 0, and 29 % and 37 % at day 14 for water samples H 1424 and W 5465, respectively (data not shown).

The results of the HPC and TCC determinations for the samples from drinking water wells are given in Figure 28.

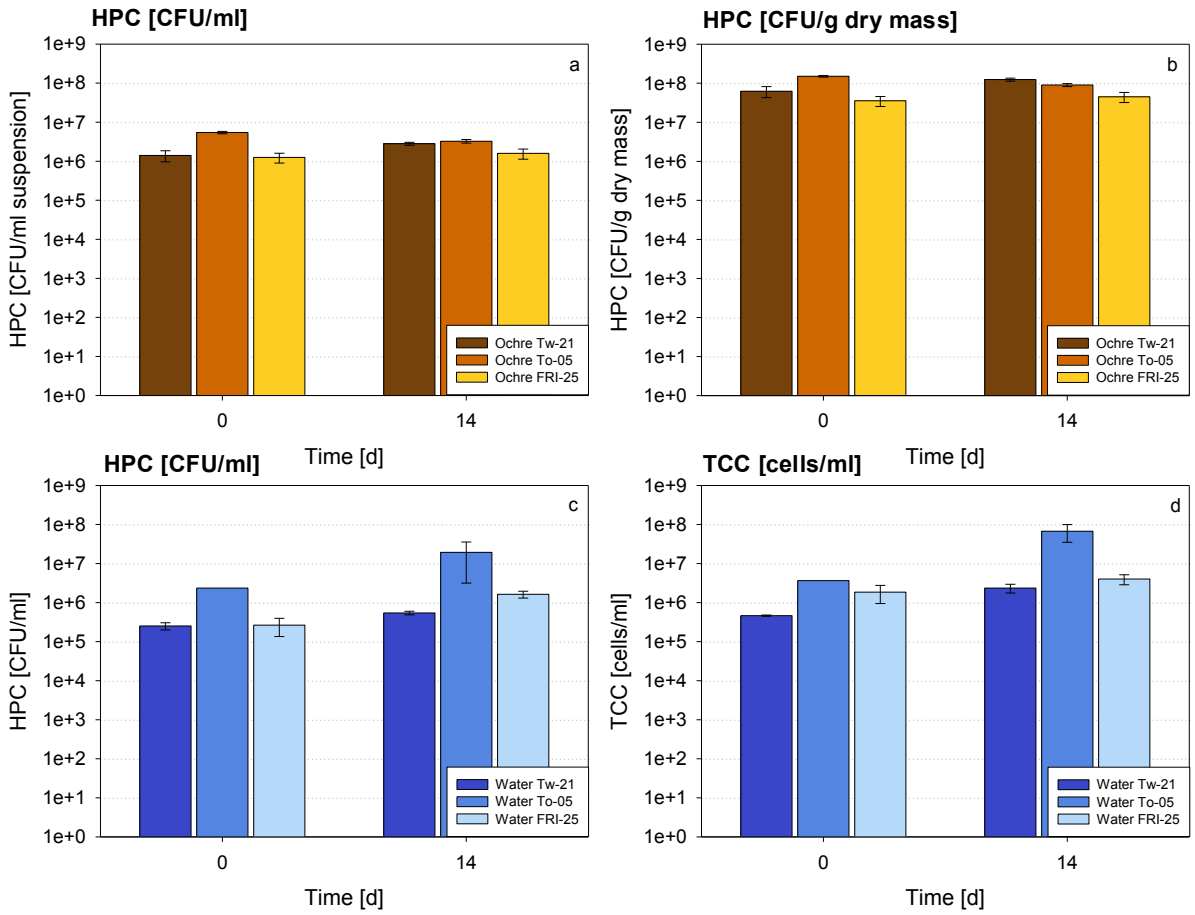

Figure 28: Heterotrphic plate count (HPC) of suspensions of ochre samples and of water samples from drinking water wells, as well as total cell count (TCC) of the water samples. Mean values with standard deviation of two consecutive runs of microcosm experiments. Values determined at the beginning (d0) and at the end (d14) of the runs. (a) HPC in CFU/ml ochre suspensions, as determined in the experiments; (b) HPC in CFU/g ochre dry mass, calculated from the values illustrates in (a) and the amount of ochre dry mass contained in the suspensions; (c) HPC of the water samples; (d) TTC of the water samples; HPC and TCC of water sample To-05 at day 0 only determined in run II.

At the beginning of the experiments the mean HPC of the ochre suspensions ranged from 1.3×10^6 – 5.4×10^6 CFU/ml which corresponds to 3.6×10^7 – 1.5×10^8 CFU/g ochre dry mass (Figure 28 a and b). During the experiments the HPC of the suspensions of ochre FRI-25 and ochre Tw-21 slightly increased by 0.1 and 0.3 log units on average, respectively, while the HPC of ochre suspension To-05 slightly decreased by 0.2 log units on average. This shows, as in the case of the ochre samples from dewatering wells, that the magnitude of the native heterotrophs contained in the different ochre samples is quite similar and also more or less constant over the course of the experiments.

The mean HPC of the water samples (Figure 28 c) Tw-21 and FRI-25, at the beginning of the experiments, was 2.5 x 10^5 CFU/ml and 2.7 x 10^5 CFU/ml, respectively. The HPC of water sample To-05, however, was 2.4 x 10^6 CFU/ml and was just determined in the second run of the experiment. Over the course of the experiments, the HPC of water samples Tw-21 and FRI-25 increased on average by 0.3 and 0.8 log units, respectively. The HPC of water sample To-05 increased only slightly by 0.1 log units during the second run of the experiment. The total cell count (TCC) in the water samples (see Figure 28 d) resulted in the following figures: On day 0, the mean TTC of water samples Tw-21 and FRI-25 was 4.6 x 10^5 cells/ml and 1.9 x 10^6 cells/ml, respectively. Whereas the TCC of the water sample To-05 at day 0 could just be determined in the second experimental run and resulted in 3.6 x 10^6 cells/ml. The TCC increased during the experiments by 0.7 and 0.4 log units on average for water samples Tw-21 and FRI-25, respectively, and by 1.0 log unit for sample To-05 in run II. These results show that these water samples from drinking water wells (from another location as the dewatering wells) contained higher amounts of indigenous microbiota, especially water sample To-05, than the water samples from dewatering wells, both total cell numbers and culturable heterotrophs. Thus, in this case, also the difference between the content of culturable heterotrophs per ml in the water microcosms, as compared to the ochre suspension microcosms, is less pronounced than for the samples from dewatering wells. In the case of sample To-05, the water microcosm at day 14 even contained a higher content of heterotrophs per ml than the respective ochre suspension. That means that the hygienically relevant bacteria spiked into the different samples are confronted with similar amounts of indigenous microbiota in ochre suspensions as well as in well water.

Over the course of the experiments, a less pronounced increase, both of total cell numbers and of culturable heterotrophs, happened in the samples from drinking water wells than in those from dewatering wells. On the other hand, the ratio of HPC to TTC, as a measure of the culturability of the cells contained in the samples, was rather high: For water samples Tw-21 and FRI-25 it was on average 54 % and 23 % at day 0, and 24 % and 42 % at day 14, respectively. Water sample To-05 yielded a HPC to TTC ratio of 65 % at day 0 of the second run and a mean ratio of 22 % at day 14.

Besides the microbiological characterisation of the ochre and water microcosms, by means of HPC and TCC, the pH-values in the microcosms were determined at the beginning and the end of the experiments. The results of the measurements showed that the pH-values both of

the ochre suspensions and the water samples were constant over the course of the experiments. Mean pH values and standard deviations of the measurements at days 0 and 14 are given in Table 24. With the exception of the samples from one dewatering well in Saxony, NW 83, which were acidic, all samples were in the neutral to slightly alkaline pH range (pH 7.0-8.5).

Table 24: pH values of suspensions of ochre samples and of water samples from dewatering wells and drinking water wells. Given are mean values and standard deviations (sd) of measurements at day 0 and day 14 of two respective runs of microcosm experiments.

	Ochre suspensions		Water samples	
Samples from	pH	sd	pH	sd
Dewatering wells				
H 1424	7.0	0.4	8.4	0.3
W 5465	7.6	0.3	7.7	0.3
NW 83	5.7	0.1	3.4	0.0
Drinking water wells				
Tw-21	7.8	0.3	8.1	0.1
To-05	7.9	0.2	8.2	0.1
FRI-25	7.6	0.2	8.5	0.1

3.5.4 Detection of target organisms in well water samples by FISH

As explained in section 3.2.1, cultivation methods usually fail to detect all bacteria present in a sample. On the one hand, the applied cultivation conditions may not be suitable, or, on the other hand, the target bacteria may have entered a viable but non-culturable (VBNC) state (cf. page 55). From all target bacteria in the present study it is known that they can enter the VBNC state (Oliver, 2010). In order to detect those, the cultivation-independent method of fluorescence in situ hybridization (FISH) was applied in addition to the cultivation methods. Unfortunately, for the ochre suspensions this could not be applied because ochre particles interfered significantly with the fluorescence microscopic quantification of the cells. Therefore, FISH results could only be achieved for well water samples but, nonetheless, these results might also be indicative for the situation within the ochre microcosms.

FISH was applied for both the native, non-inoculated and the spiked well water samples from the microcosm experiments. For water sample H 1424, however, FISH was only performed with probes Colinsitu, Psae 16S-182, LEGPNE1 and Kpn, but not with the probes Efs 130 and AERBOMO, because the native, non-inoculated H 1424 water sample yielded no positive result for the probes applied and the spiked samples contained too few target cells. Therefore, in the following microcosm experiments the samples were spiked with a higher initial concen-

tration of target bacteria (1 x 10^8 cells/ml) than in the experiments using samples H 1424 (1 x 10^6 cells/ml).

The recovery rates of the different target bacteria by FISH were determined for the day 0 microcosm well water samples which were spiked with 1 x 10^8 cells/ml. The results are given in Table 25 complemented, for comparison, by the recovery rates of the cultivation techniques applied to the same samples.

Table 25: Recovery rates of FISH using the denoted probes for quantification of target organisms in well water. Given are the mean recovery rates (ratio of FISH-positive cells or CFU detected to initial cell concentration adjusted within the spiked samples) for day 0 microcosm samples which were spiked with 1 x 10^8 cells/ml and the maximal recovery rate found within these samples. Mean and maximal recovery rates of the cultivation techniques applied to the same samples are given for comparison. Colilert, Colilert®-18/Quanti-Tray®/2000; Spread plate methods using: CEA, Chromocult® Enterococci-Agar; CN, Pseudomonas CN agar; ADA, Ampicillin-dextrin agar; GVPC, GVPC agar.

	Recovery rate [%] FISH			Recovery rate [%] Cultivation		
Target	FISH probe	Mean	Maximum	Method	Mean	Maximum
E. c.	Colinsitu	82	Complete recovery	Colilert	Complete recovery	
K. p.	Kpn	86	Complete recovery	Colilert	59	Complete recovery
E. f.	Efs 130	58	67	CEA	39	58
P. a.	Psae 16S-182	92	Complete recovery	CN	26	58
A. h.	AERBOMO	77	96	ADA	17	34
L. p.	LEGPNE1	52	69	GVPC	15	37

As expected, the recovery rates of FISH were in most cases considerably higher than those of the cultivation techniques. This is in accordance to the finding that for oligotrophic to mesotrophic aquatic habitats usually direct microscopic counts exceed plate counts significantly (AMANN et al. 1995; cf. 3.2.1). Only in the case of the detection of *E. coli*, the recovery rates of FISH and the MPN method Colilert®-18/Quanti-Tray®/2000 were quite similar, or even higher for the cultivation technique.

Native, non-inoculated well water samples

FISH-positive results for the native, non-inoculated well water samples from these microcosm experiments are given in Table 26. Of the five water samples tested, three samples were FISH-positive for probe Colinsitu and probe LEGPNE1, respectively, four for Efs 130 and Psae 16S-182, respectively, and all samples were FISH-positive for probes Kpn and AERBOMO. Numbers of target cells were in the range of 6.1 x 10^1 and 1.3 x 10^6 cells/ml.

Table 26: FISH-positive results for native, non-inoculated well water samples at day 0 of the microcosm experiments. Two consecutive experimental runs (I, II) of 14 days each were conducted with the samples, respectively. TCC, total cell count. Run I W. NW 83 and W. To-05: TCC could not be defined; therefore it was not possible to quantify the FISH-positive cells. W. NW 83 run I: FISH-positive cells with probe Efs 130 and AERBOMO; W. To-05 run I: FISH-positive cells with probe Efs 130, LEGPNE1, Kpn and AERBOMO. Target organisms in brackets: *E. c.*, *E. coli*; *E. f.*, *E. faecalis*; *P. a.*, *P. aeruginosa*; *L. p.*, *L. pneumophila*; *K. p.*, *K. pneumoniae*; *A. h.*, *A. hydrophila*.

		[Target cells/ml] with probe						[Cells/ml]
Sample	Day 0 Run	Colinsitu (*E. c.*)	Efs 130 (*E. f.*)	Psae 16S-182 (*P. a.*)	LEGPNE1 (*L. p.*)	Kpn (*K. p.*)	AERBOMO (*A. h.*)	TCC
W. W 5465	I	1.2×10^4	-	-	-	-	6.2×10^2	2.3×10^4
	II	-	-	-	-	8.8×10^3	1.2×10^4	9.2×10^4
W. NW 83	I	-	p	-	-	-	p	n.d.
	II	-	5.5×10^2	6.1×10^1	2.7×10^2	8.5×10^2	-	2.5×10^3
W. Tw-21	I	5.3×10^4	1.9×10^5	4.3×10^4	-	5.8×10^4	-	4.8×10^5
	II	-	5.1×10^4	3.5×10^4	-	6.1×10^4	3.2×10^4	4.5×10^5
W. To-05	I	-	p	-	p	p	p	n.d.
	II	3.4×10^5	4.0×10^5	1.3×10^6	5.1×10^5	1.2×10^6	2.3×10^5	3.6×10^6
W. FRI-25	I	-	1.0×10^5	7.0×10^4	1.4×10^5	8.8×10^4	4.6×10^4	9.4×10^5
	II	-	7.8×10^5	5.9×10^5	2.6×10^5	2.3×10^5	4.3×10^4	2.8×10^6

By means of cultivation methods the same water samples yielded only three positive results for the different target bacteria. Water samples W 5465 and Tw-21 resulted in 1.5 and 2 CFU/100 ml of *Aeromonas* spp., respectively, whereas in water sample FRI-25 coliform bacteria (1 MPN/100 ml) were detected by Colilert®-18/Quanti-Tray®/2000.

Spiked well water samples

Results for the spiked well water samples from the microcosm experiments, in which the samples were initially inoculated with target bacteria concentrations of 1×10^8 cells/ml, gained by FISH and, for comparison, also by cultivation are given in the next five figures.

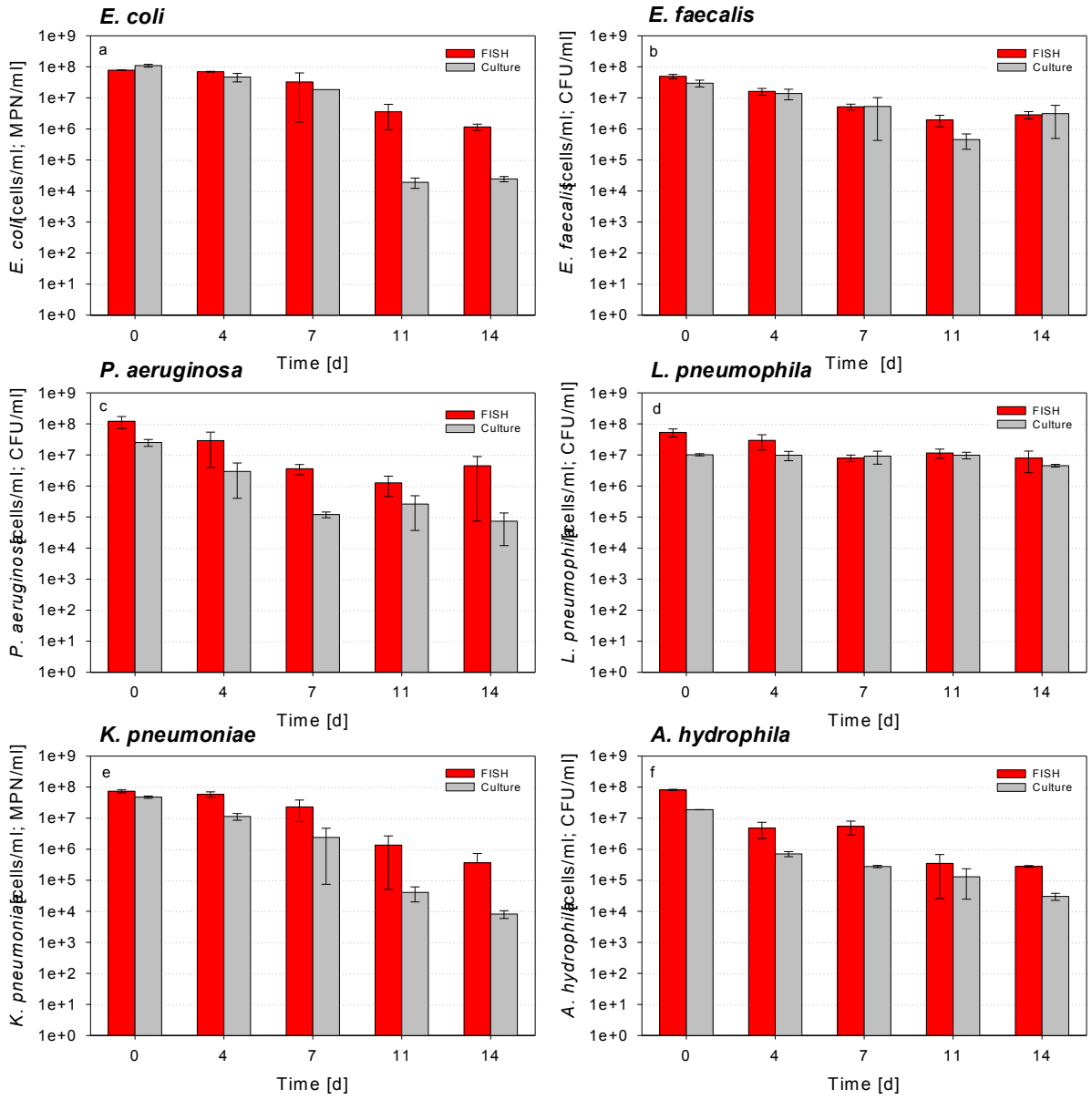

Figure 29: Detection of target organisms in Water W 5465 over time by fluorescence in situ hybridization (FISH) or cultivation (Culture). Mean values with standard deviations of two consecutive runs of microcosm experiments. Water sample from a dewatering well spiked with target organisms, initial concentration at day 0: 1 x 10^8 cells/ml.

In general, FISH yielded equally high or mostly higher results than the cfu numbers of the same sample. Major differences between the results of the methods were apparent in case of a strong decrease of MPN or CFU over time, for the decrease of FISH-positive cells was much less pronounced, or numbers of FISH-positive were even constant over time. This was especially true for the microcosm experiment using water sample NW 83 (Figure 30). For all target organisms numbers of FISH-positive cells were higher than numbers of MPN or CFU, especially over the course of time.

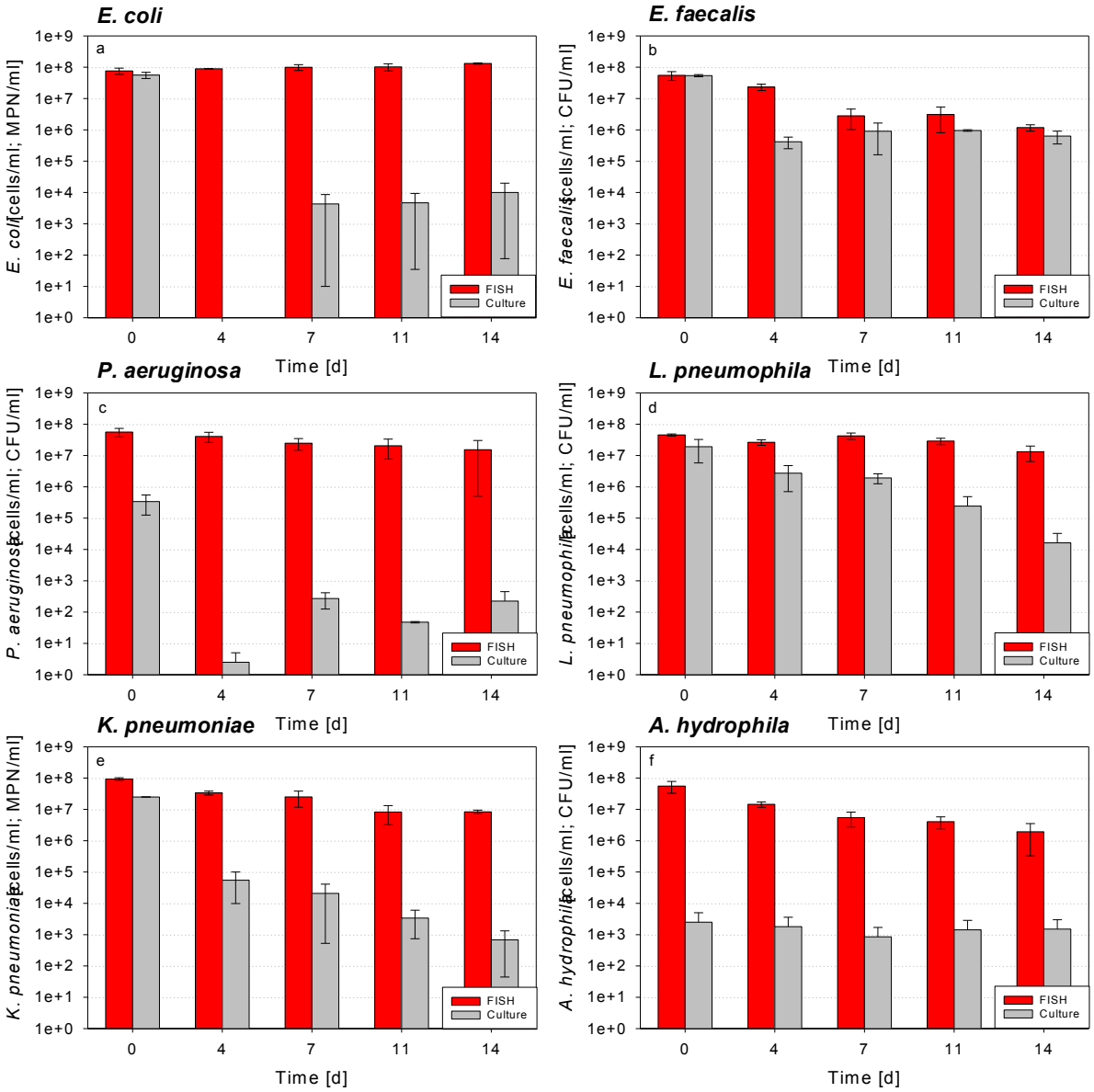

Figure 30: Detection of target organisms in Water NW 83 over time by fluorescence in situ hybridization (FISH) or cultivation (Culture). Mean values with standard deviations of two consecutive runs of microcosm experiments. Water sample from a dewatering well spiked with target organisms, initial concentration at day 0: 1 x 10^8 cells/ml.

For *E. coli*, *P. aeruginosa* and *K. pneumoniae* FISH yielded ≥ 4 orders of magnitude higher results at day 14 of the experiments than the respective cultivation method (Figure 30 a, c, e) and for *L. pneumophila* and *A. hydrophila* this difference was about 3 orders of magnitude, considering the respective mean values from the two experimental runs (Figure 30 d, f). Regarding the single runs, the difference for *A. hydrophila* is even higher, because in run I *A. hydrophila* was no longer detected by cultivation from day 4 onwards and in run II it was not detected at day 0 (cf. Figure 23 d).

Only in the case of *E. faecalis*, the difference between the results gained by FISH as compared to the ones gained by cultivation was not that pronounced as for the other target organisms. But *E. faecalis* was also the target organisms which showed the smallest decrease in numbers of colony forming units over the course of time in the microcosm experiments using water sample NW 83 (Figure 30 b).

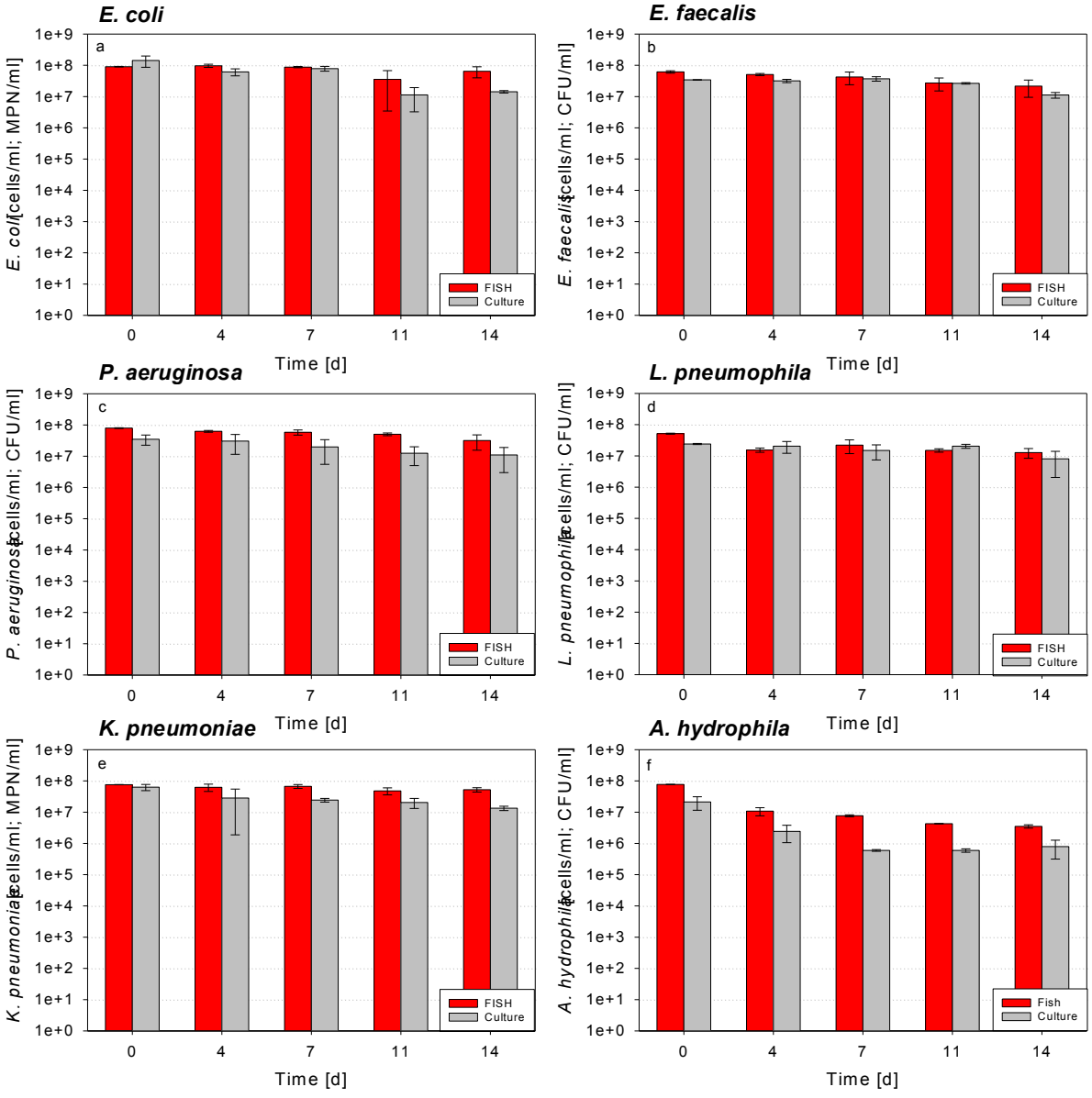

Figure 31: Detection of target organisms in Water Tw-21 over time by fluorescence in situ hybridization (FISH) or cultivation (Culture). Mean values with standard deviations of two consecutive runs of microcosm experiments. Water sample from a drinking water well spiked with target organisms, initial concentration at day 0: 1 x 10^8 cells/ml.

Regarding the other microcosm experiments, numbers of *E. coli* and *K. pneumoniae* gained by FISH were significantly higher (differences of up to 1-3 log units) in water samples W 5465 and To-05 (Figure 29 a, e and Figure 32 a, e), particularly from day 4 onwards, than the results from cultivation methods; as well as numbers of *P. aeruginosa*, *L. pneumophila* or *A. hydrophila* in water samples W 5465, To-05, and Tw-21 and FRI-25, respectively (Figure 29 c, Figure 32 d, Figure 31 f and Figure 33 f). Otherwise, results by cultivation or by FISH were quite similar, with just a bit higher yield by the latter.

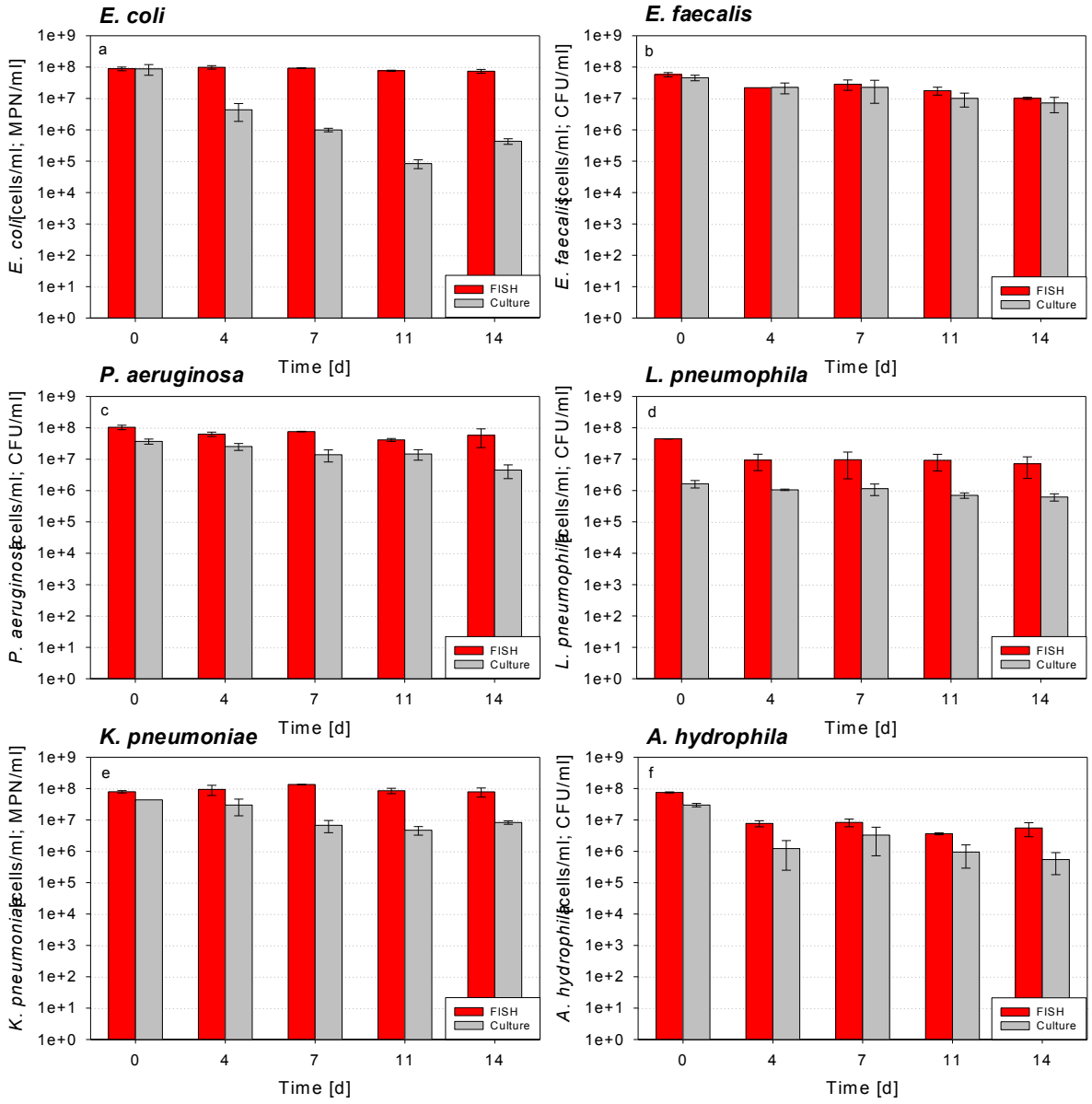

Figure 32: Detection of target organisms in Water To-05 over time by fluorescence in situ hybridization (FISH) or cultivation (Culture). Mean values with standard deviations of two consecutive runs of microcosm experiments. Water sample from a drinking water well spiked with target organisms, initial concentration at day 0: 1 x 10^8 cells/ml.

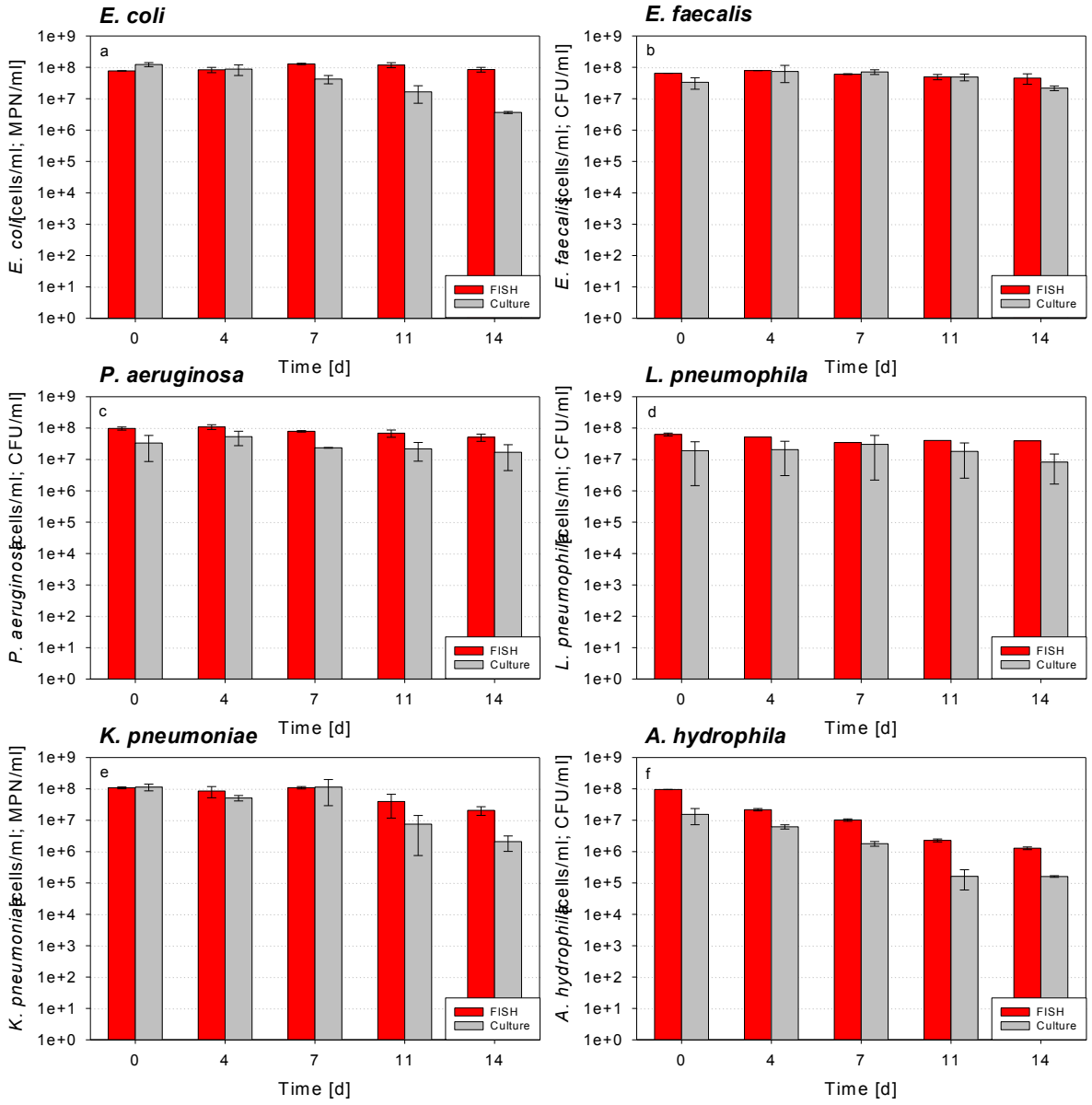

Figure 33: Detection of target organisms in Water FRI-25 over time by fluorescence in situ hybridization (FISH) or cultivation (Culture). Mean values with standard deviations of two consecutive runs of microcosm experiments. Water sample from a drinking water well spiked with target organisms, initial concentration at day 0: 1 x 10^8 cells/ml.

Altogether, the results show that for all target organisms except *E. coli* the recovery rates of FISH are considerably higher than those of the respective cultivation method. Furthermore, it turned out, as expected, that in case of a sharp drop in MNP or CFU/ml, over the course of the microcosm experiments, the decline in numbers of FISH-positive cells was much less pronounced, or those numbers were even constant over time.

3.5.5 Detection of *L. pneumophila* and *P. aeruginosa* in ochre suspension and well water samples by qPCR

Since a direct fluorescence microscopic quantification of target bacteria in ochre suspension was not possible due to the significant interference of the ochre particles with the detection method, qPCR as another culture-independent quantification method was applied to complement the traditional cultivation methods (cf. 3.2.1 and 3.5.4). However, the limited time within the project did not allow for the development of a qPCR method for each target organism. Therefore, qPCR results were only gained for *L. pneumophila*, using a commercially available kit (iQ-CheckTM Quanti *L. pneumophila* Kit, BioRad), and for *P. aeruginosa*, according to the TaqMan probe assay described by FRÖSLER (2011) and published in TEWES (2012).

Recovery rates of qPCR (ratio of GU [genome units] detected to initial cell concentration adjusted within the spiked samples) for quantification of *L. pneumophila* or *P. aeruginosa* in different ochre suspensions and well water samples are presented in Table 27 together with the recovery rates of cultivation and FISH, within these samples, for comparison. These results show that the recovery rate of qPCR is quite diverse for the different samples, as well as for the different target bacteria. Not in all cases the recovery rate of qPCR was higher as the one of the respective cultivation method, as actually expected (cf. last paragraph of section 4.4.4). This is especially true for the recovery rate of *P. aeruginosa*-qPCR, which was only for ochre sample NW 83 higher than the recovery rate of the cultivation method (spread plate methods using Pseudomonas CN agar). For *P. aeruginosa*-qPCR, particularly for the water samples, the recovery rate is extremely low, only 0.3 to 3 %, and also for the ochre samples it is just 6 to 19 %. In comparison, the recovery rate of *L. pneumophila*-qPCR is indeed higher, 24 % to complete recovery for the ochre samples and 8 % to complete recovery for the water samples. But also in this case for some samples, e.g. ochre samples Tw-21 or To-05 and water sample NW 83, it is with 24 %, 34 % and 8 % not as high as expected.

Table 27: Recovery rates of qPCR, cultivation and FISH for quantification of *L. pneumophila* or *P. aeruginosa* at day 0 in ochre suspensions and well water samples from microcosm experiments. Given are the recovery rates (ratio of GU [genome units], CFU or FISH-positive cells detected to initial cell concentration adjusted within the spiked samples) for the samples for which the results are graphically displayed in the next four figures. R., Recovery. Culture, Spread plate methods using: GVPC, GVPC agar; CN, Pseudomonas CN agar.

	Recovery rate [%]				
Sample	**W 5465**	**NW 83**	**Tw-21**	**To-05**	**FRI-25**
L. pneumophila					
Ochre					
qPCR	68	Complete R.	24	34	57
Culture (GVPC)	12	45	20	1	99
Water					
qPCR	Complete R.	8	60	Complete R.	Complete R.
Culture (GVPC)	9	33	25	1	37
FISH	68	42	54	44	56
P. aeruginosa					
Ochre					
qPCR	6	19	13	12	12
Culture (CN)	7	1	57	29	30
Water					
qPCR	3	0.3	1	3	1
Culture (CN)	32	1	48	30	58
FISH	71	39	77	87	86

The qPCR methods were applied for the same samples from the microcosm experiments as FISH (cf. section 3.5.4), both the native, non-inoculated and the spiked well water samples, and for the corresponding ochre samples. qPCR results for the native, non-inoculated ochre and well water samples are given in Table 28.

Table 28: qPCR results for native, non-inoculated ochre and well water samples. GU, genome units. It should be noted that the results for the water samples are given in GU per 100 ml. *L. pneumophila* and *P. aeruginosa* were not detected by cultivation (spread plate methods using GVPC agar or Pseudomonas CN agar) within these samples.

Ochre sample	*L. pneumophila* [GU/g dry mass]	*P. aeruginosa* [GU/g dry mass]
W 5465	8.0×10^3	2.1×10^2
NW 83	3.2×10^2	1.2×10^4
Tw-21	-	1.6×10^2
To-05	6.8×10^3	3.4×10^1
FRI-25	1.3×10^3	-
Tw-22	-	-
Tw-24	1.5×10^3	-
Well water sample	***L. pneumophila* [GU/100 ml]**	***P. aeruginosa* [GU/100 ml]**
Tw-21	3.8×10^1	1.3×10^1
FRI-25	2.8×10^1	2.1×10^0

Results for the spiked samples from the microcosm experiments, in which the samples were initially inoculated with target bacteria concentrations of 1 x 10^8 cells/ml, gained by qPCR and, for comparison, also by cultivation and FISH are illustrated in the next four figures. Figure 34 and Figure 35 show the results for the samples spiked with *L. pneumophila*; the first one for the ochre and water samples sampled from dewatering wells and the second for the ones from drinking water wells. In the samples from dewatering well W 5465, numbers of genome units detected by qPCR were about one order of magnitude higher than the numbers of CFU, and the GU numbers were also higher than the FISH positive cells (Figure 34). However, in the samples from well NW 83 this was quite different: In this case only for ochre suspension sample d 0 and the water samples d 7 and d 14 GU numbers were higher than numbers of CFU, and for all water samples FISH yielded higher results than qPCR (Figure 35).

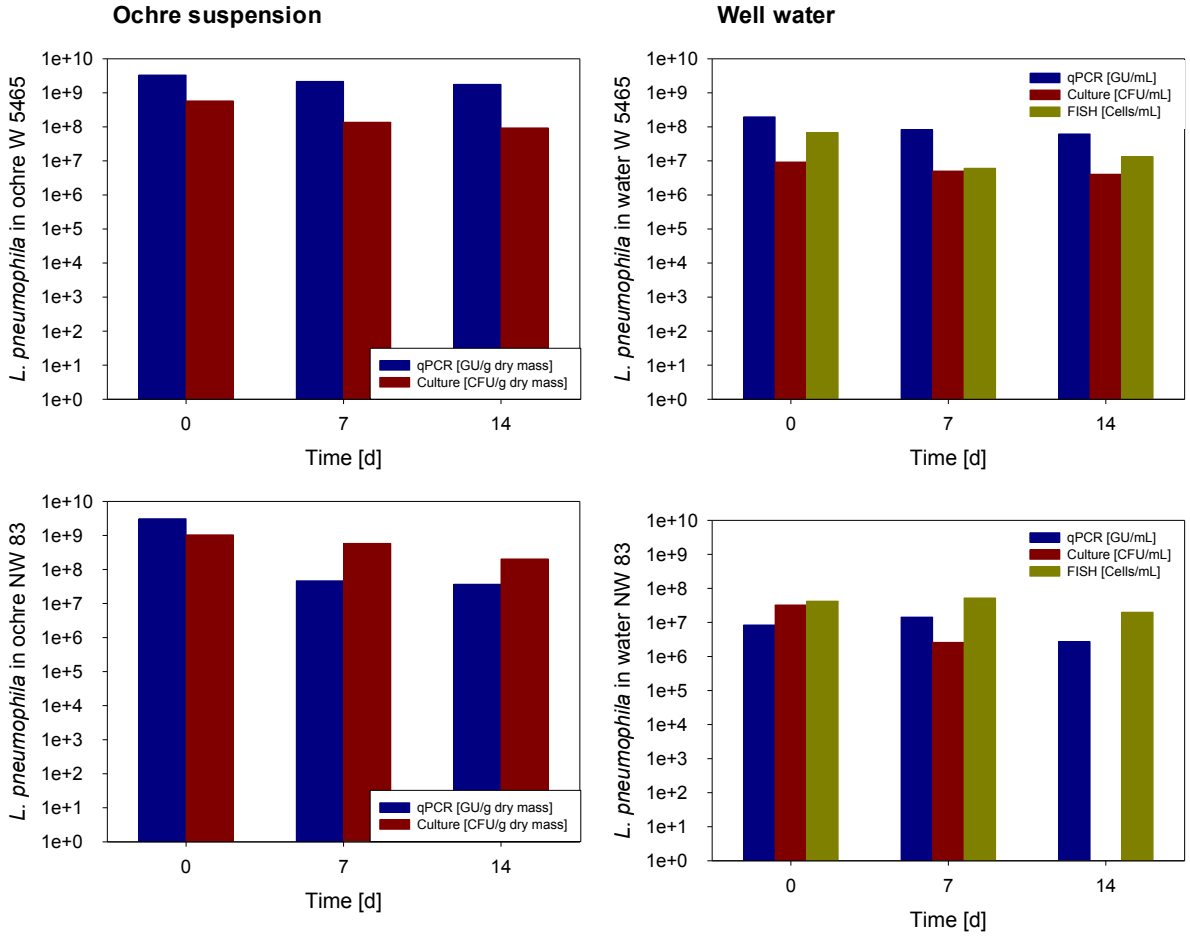

Figure 34: Detection of *L. pneumophila* in spiked ochre suspensions by qPCR and cultivation, and in spiked well samples by qPCR, cultivation and FISH; set of samples from microcosm experiments (cf. 3.5.3) originally taken from dewatering wells. Culture: Spread plate method using GVPC agar. Initial cell concentration: Ochre W 5465, 4.9 x 10^9 cells/g dry mass; Ochre NW 83, 2.9 x 10^9 cells/g dry mass; Water samples, 1 x 10^8 cells/ml.

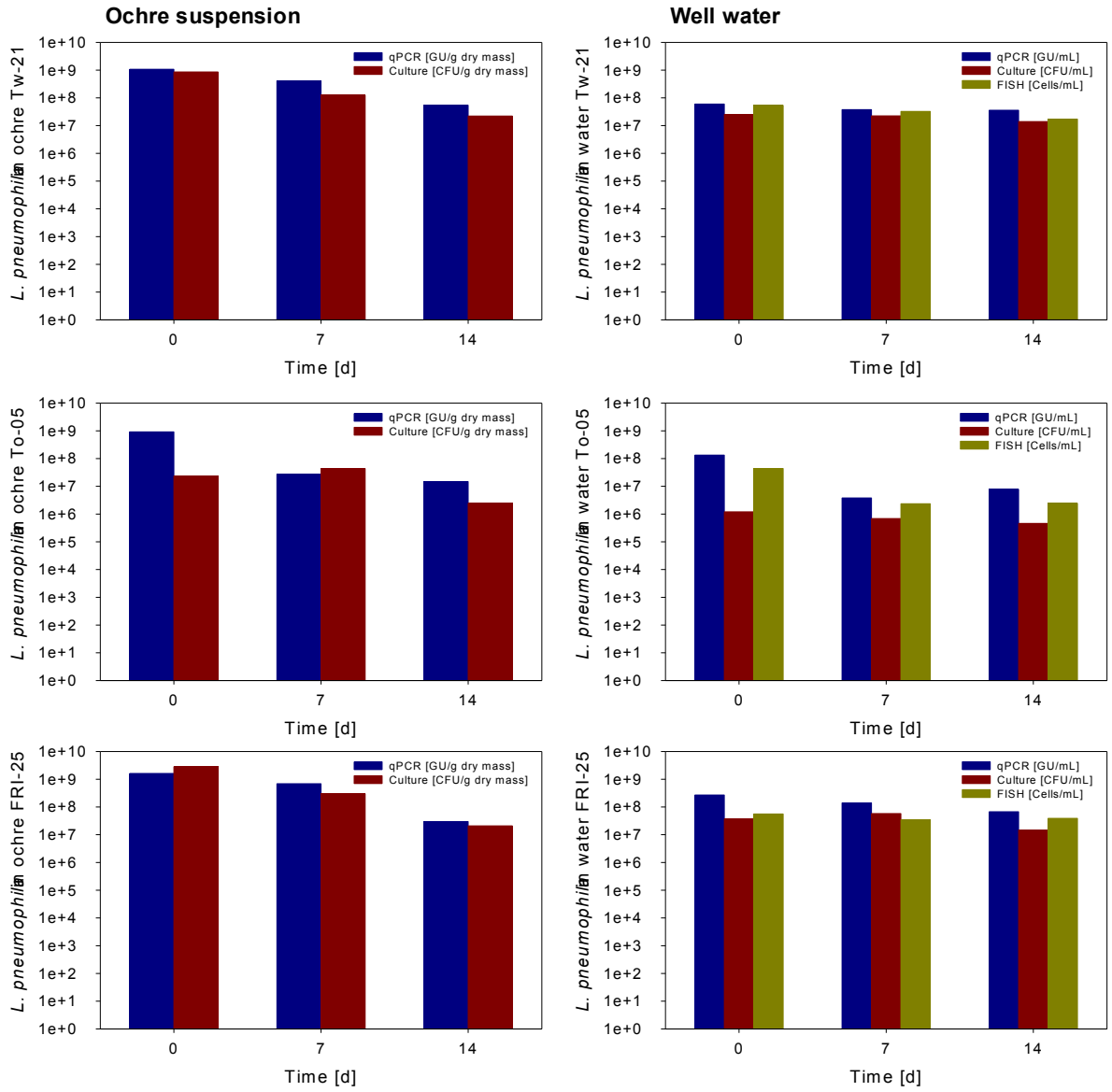

Figure 35: Detection of *L. pneumophila* in spiked ochre suspensions by qPCR and cultivation, and in spiked well samples by qPCR, cultivation and FISH; set of samples from microcosm experiments (cf. 3.5.3) originally taken from drinking water wells. Culture: Spread plate method using GVPC agar. Initial cell concentration: Ochre Tw-21, 4.4 x 10^9 cells/g dry mass; ochre To-05, 2.7 x 10^9 cells/g dry mass; ochre FRI-25, 2.8 x 10^9 cells/g dry mass; Water samples, 1 x 10^8 cells/ml.

For the set of microcosm samples from drinking water wells, detection of *L. pneumophila* by qPCR resulted only for ochre and water samples To-05 d 0 in higher GU numbers compared to numbers of CFU; for the other samples qPCR yielded results in a similar range as cultivation and FISH, or only slightly higher ones (Figure 35).

Furthermore, *P. aeruginosa* qPCR showed even more unexpected results as *L. pneumophila* qPCR, especially for the water samples, as compared to the ones gained by cultivation or FISH. Given are these results in Figure 36 for the microcosm samples originally taken from

dewatering wells and in Figure 37 for the samples from drinking water wells. Except for the ochre suspension samples W 5465 d 14 and NW 83 (d 0, d 7, d 14) and water samples NW 83 d 7 and d 14, *P. aeruginosa* qPCR resulted in smaller GU numbers than numbers of CFU for all other samples. Likewise, numbers of GU were significantly lower than numbers of FISH positive cells, up to four orders of magnitude, for all water samples from dewatering wells and drinking water wells. This was totally unexpected, since normally culture-independent methods detect higher numbers of target organisms than cultivation, and the two culture-independent methods, qPCR and FISH, were expected to yield similar results. Therefore, these results highlight the need for further validation and adjustment of the qPCR methods for ochre and well water samples.

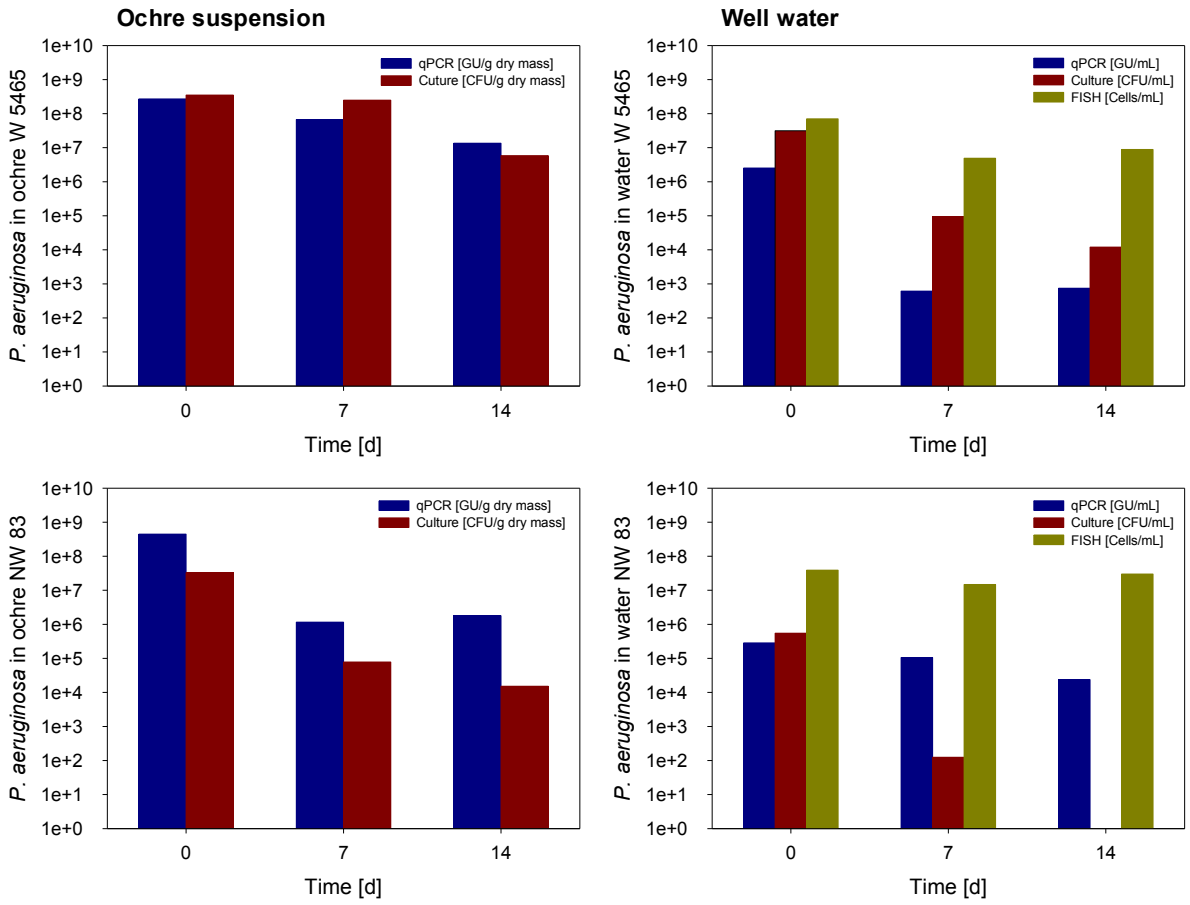

Figure 36: Detection of *P. aeruginosa* in spiked ochre suspensions by qPCR and cultivation, and in spiked well samples by qPCR, cultivation and FISH; set of samples from microcosm experiments (cf. 3.5.3) originally taken from dewatering well. Culture: Spread plate method using Pseudomonas CN agar. Initial cell concentration: Ochre W 5465, 4.9 x 10^9 cells/g dry mass; ochre NW 83, 2.9 x 10^9 cells/g dry mass; Water samples, 1 x 10^8 cells/ml.

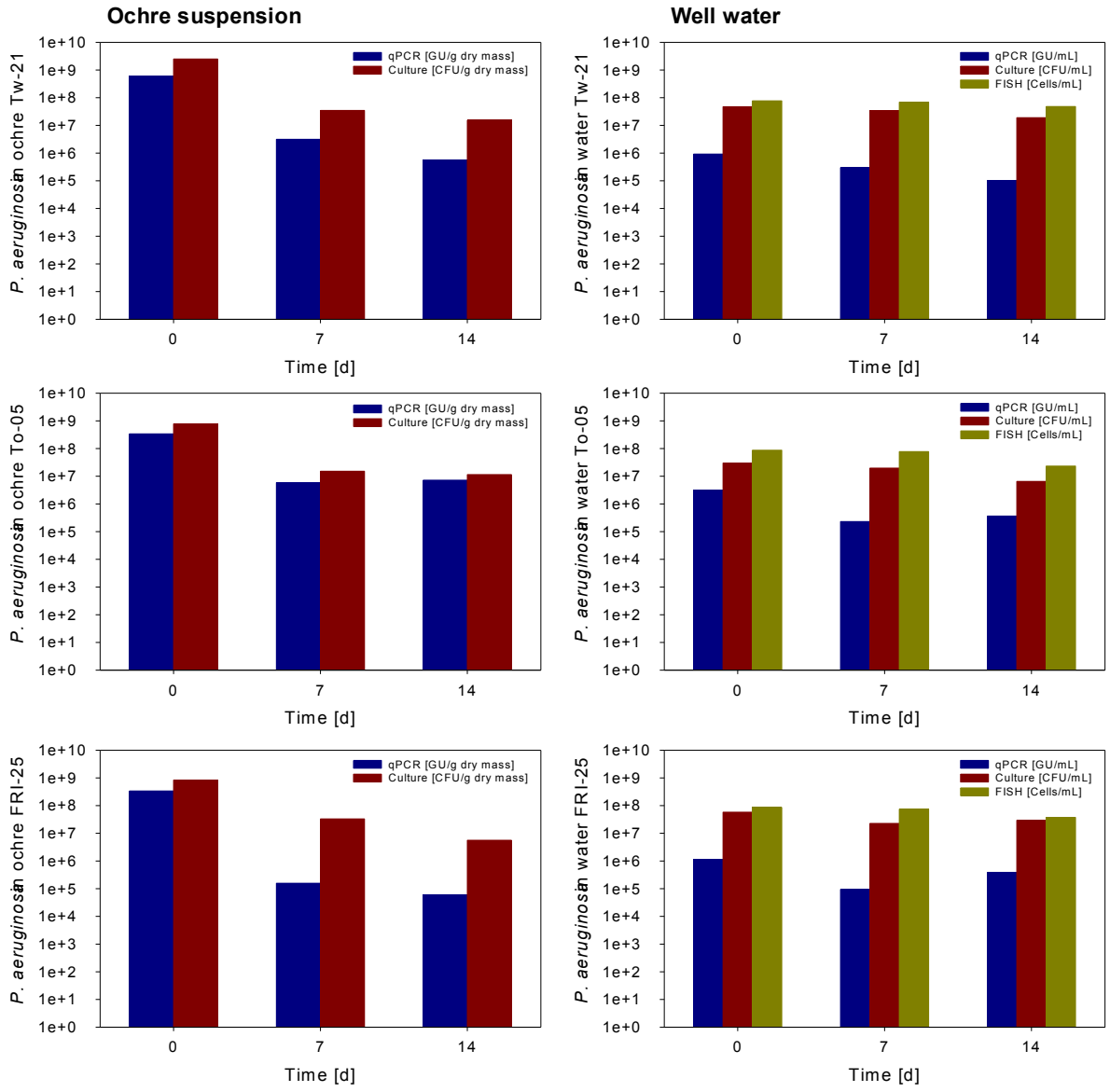

Figure 37: Detection of *P. aeruginosa* in spiked ochre suspensions by qPCR and cultivation, and in spiked well samples by qPCR, cultivation and FISH; set of samples from microcosm experiments (cf. 3.5.3) originally taken from drinking water wells. Culture: Spread plate method using Pseudomonas CN agar. Initial cell concentration: Ochre Tw-21, 4.4 x 10^9 cells/g dry mass; ochre To-05, 2.7 x 10^9 cells/g dry mass; ochre FRI-25, 2.8 x 10^9 cells/g dry mass; Water samples, 1 x 10^8 cells/ml.

3.6 Column experiments – survival of hygienically relevant bacteria attached to ochre under flow-through conditions

In the microcosm experiments (cf. section 3.5), indicator bacteria of faecal and non-faecal contamination and opportunistic pathogens were spiked into suspensions of ochre samples from different wells, followed by the determination of their concentration over time, to check the potential of those bacteria to survive in an ochre containing matrix. In the column experiments, described in the following section, the survival potential of the target bacteria was studied under flow-through conditions attached to ochre. These experiments were conducted,

since those conditions more closely resemble the conditions within a well and the strategy of the present study was to simulate the contamination of an ochre-incrusted well by hygienically relevant bacteria to elucidate if such incrustations can act as sink and source for pathogens.

Horizontally arranged columns, half-filled with ochre, were used to test the survival of target organisms attached to ochre under flow-through conditions. In preliminary tests, the volume needed to fill the space above the ochre (i.e. the water phase of the columns) and the period of time needed for an exchange of the water phase were determined. For that purpose a coloured solution ($KMnO_4$; 2 mg/ml) was injected into the columns and the discharge from the columns was observed. The tests revealed that a volume of 10 ml has to be injected into the columns to fill the water phase completely, even though the calculated volume of the water phase of a column filled with ochre in the lower half was only about 5 ml. A complete exchange of the water phase took one hour, at a flow-rate of 18 ml/h. Therefore, one hour after start of flow-through was set as the first sampling point in the column experiments to test the survival of the target organisms attached to ochre under flow-through conditions.

3.6.1 Inoculation of ochre with target organisms within the columns

In an initial series of experiments the ochre was inoculated with target organisms within the columns. That is, columns were filled in the lower half with ochre from a drinking water well, were assembled and set up, and then 10 ml bacterial suspension (prepared in synthetic groundwater; one target organism at a time, 1 x 10^8 cells/ml) were injected into each column. This suspension remained static in the columns overnight (except for *L. pneumophila*; stagnation = 5 h); afterwards the flow-through was started.

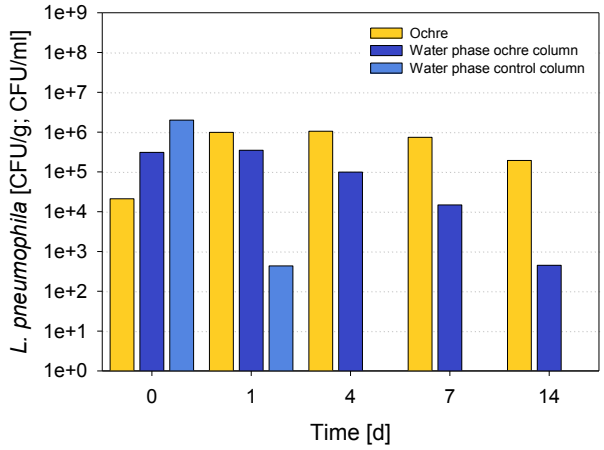

Figure 38: Survival of *L. pneumophila* attached to ochre under flow-through conditions – inoculation of the ochre within the columns. In addition to the columns half-filled with ochre, a control column without ochre was run.

As a control, an additional column without ochre was run in these experiments. Checking the water phase of the control column for target organisms revealed a complete discharge of *L. pneumophila* over the course of the experiment, but not for the other target bacteria. *L. pneumophila* was no longer detected in the water phase of the column without ochre from day 4 onwards (Figure 38). In contrast, *K. pneumoniae*, *E. faecalis*, *P. aeruginosa* and *A. hydrophila* each were detected in the water phase of the column without ochre for up to 14 days, i.e. over the whole course of the experiments (Figure 39): In the majority of cases numbers of target organisms detected in the water phase of the columns without ochre were equally high or even higher than those in the water phase of the columns with ochre, especially for *K. pneumoniae* (Figure 39 a). This led to the assumption that the target organisms had attached to the inner surface of the columns which thus functioned as a reservoir for the organisms. Therefore swab samples of different parts of the columns and the other experimental setup (cf. Figure 12) were analysed for target organisms by cultivation.

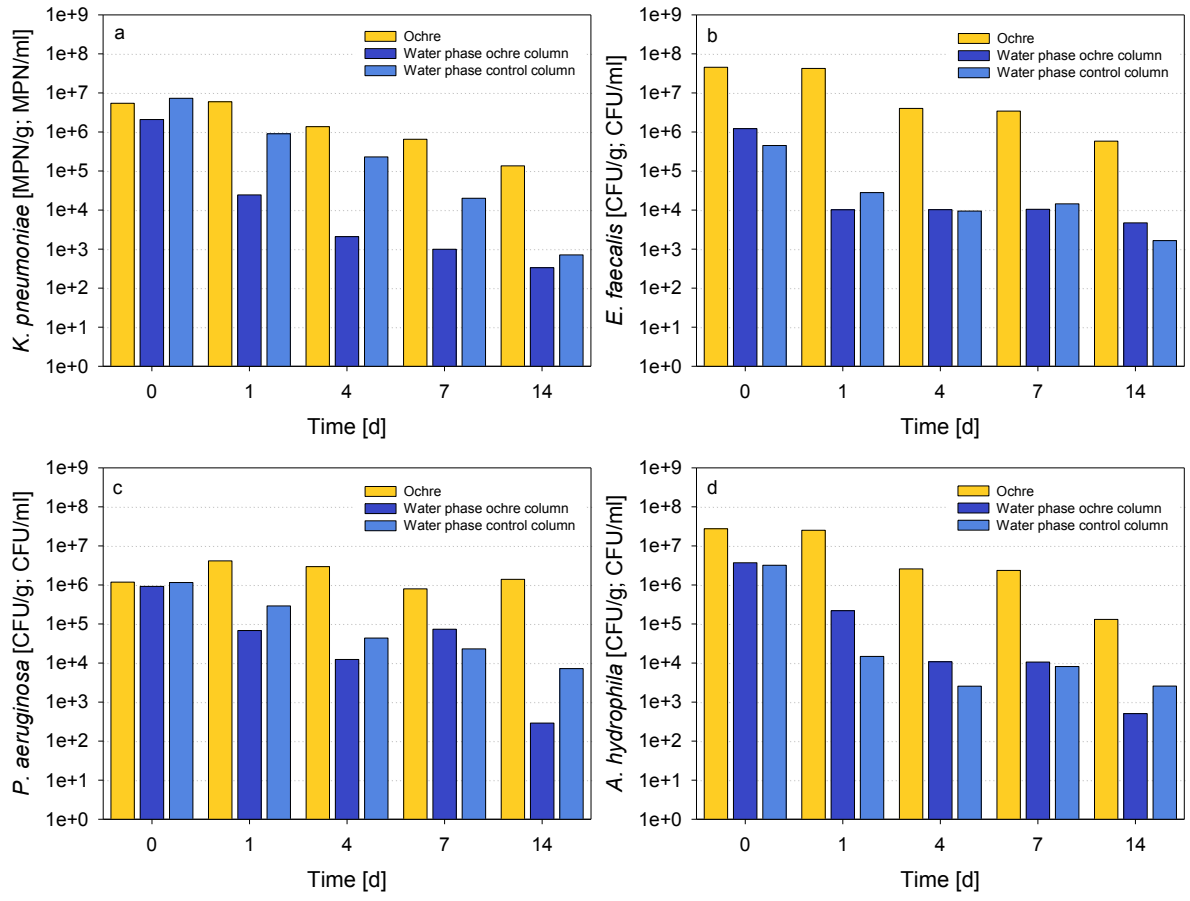

Figure 39: Survival of target organisms attached to ochre (O. FRI-25) under flow-through conditions – inoculation of the ochre within the columns. In addition to the columns half-filled with ochre, a control column without ochre was also run in each of the experiments.

For *K. pneumoniae*, *E. faecalis* and *P. aeruginosa* numbers of target organisms cultured from the swap samples of the inner surface of the column without ochre were in the range of 1.0 x 10^6 to 6.1 x 10^6 MPN or CFU/sample, at days 0-5 and day 14 after start of flow-through, respectively (Figure 40 a-c). For *A. hydrophila* a number of 1.3 x 10^4 CFU was detected in this swab sample at day 14 (Figure 40 d). In the water phase of the columns numbers of target organisms were in the range of 4.6 x 10^5 to 2.1 x 10^6 CFU or MPN/sample at the beginning of the experiments (Figure 40 a, b) and in the range of 1.0 x 10^4 to 2.9 x 10^4 CFU/sample at day 14 (Figure 40 c, d). *K. pneumoniae*, *E. faecalis* and *P. aeruginosa* were also detected in the influent capillary of the column, in numbers of 1.1 x 10^1 MPN/sample for the first one, and 7.6 x 10^3 to 4.7 x 10^4 CFU/sample for the latter both, which were also found in the swap sample of the influent tube connected to the capillary (Figure 40 a-c). No target organisms were detected in the synthetic ground water from the storage vessel.

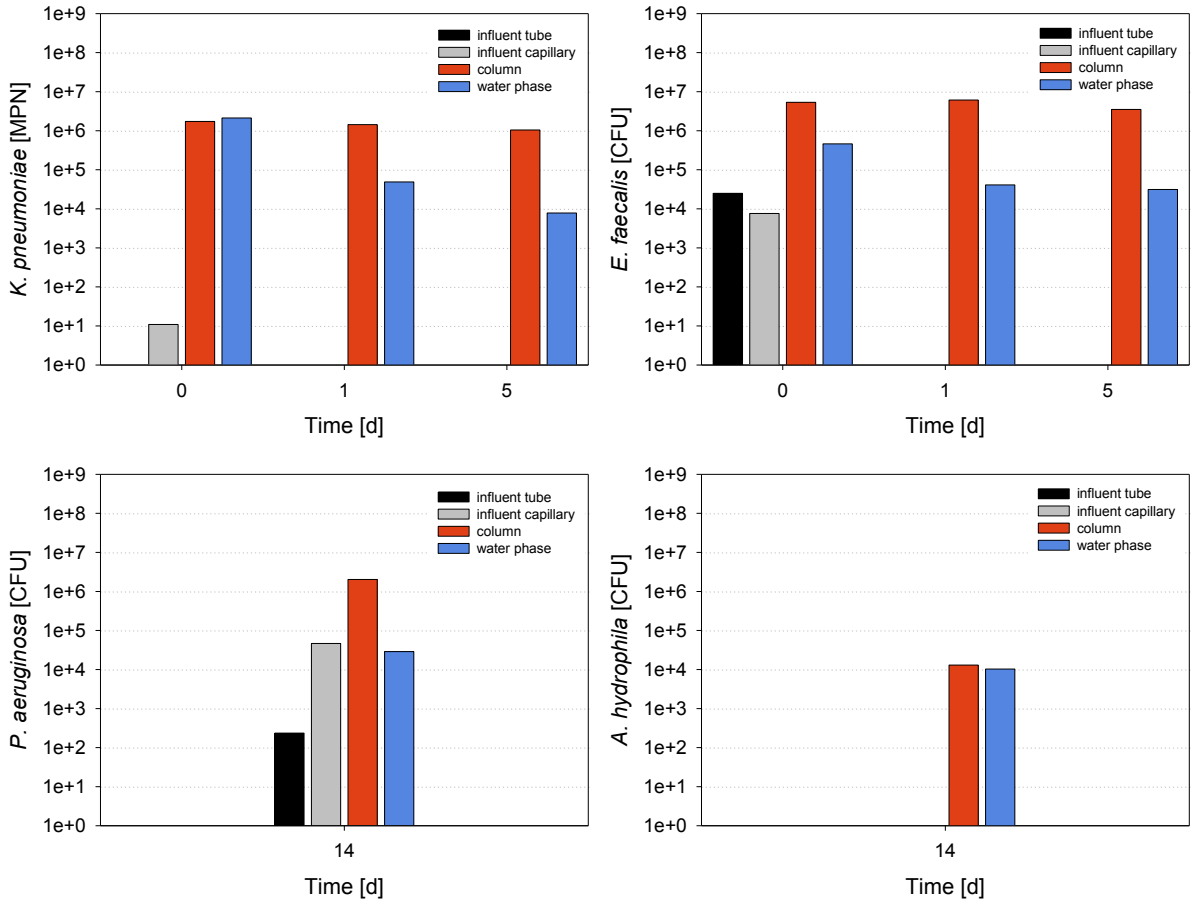

Figure 40: Test for target organisms in different parts of the experimental setup – experiments with a control column without ochre. Analysed were swap samples from the inner surface of the influent tube and capillary and of the column without ochre, and also the water phase from this column and the synthetic ground-water (sGW) in the storage vessel (cf. Figure 12). No target organisms were detected in the samples from the storage vessel. Numbers of target organisms given in the diagrams refer to the respective total surface or total volume sampled.

Apart from these findings, the series of experiments also demonstrated that all target organisms survived in a culturable state in ochre, which had been in contact with those organisms, even under flow-through conditions for up to 14 days (Figure 39). Furthermore, target organisms can also be found in the water phase of the columns over the course of the experiment (Figure 39), in numbers of about $10^2 – 10^6$ MPN or CFU/ml, even though it is not possible to tell if those target organisms arise from the ochre or other surfaces of the system. Therefore, the method of inoculation of the ochre with target organisms was modified. The test organisms were no longer injected into columns containing ochre, but ochre was inoculated with target organisms outside the columns. This ochre, with target organisms attached to it, was inserted into the columns and exposed to flow-through conditions for up to 14 days.

3.6.2 External inoculation of ochre with target organisms

In this series of experiments, ochre was inoculated with target organisms outside the columns and then this ochre, with target organisms attached to it, was inserted into columns to test the survival of the organisms under flow-through conditions for up to 14 days. This external inoculation of the ochre was done to avoid the attachment of target organisms to other surfaces of the system, as it happened when bacterial suspensions were injected into the ochre containing columns for inoculation of the ochre (cf. section 3.6.1), and to know that all target bacteria within the system originate from the ochre. Results of the different runs are presented in Figure 41 and Figure 42. In Figure 41 numbers of target organisms in ochre and in the water phase of the respective column are presented in MPN or CFU/g ochre wet mass and in MPN or CFU/ml, respectively, to illustrate the amount of target organisms released from the respective ochre sample into the water phase. In Figure 42, however, only the results gained for the ochre samples are given in $\log(N/N_0)$ and compared to results from a microcosm experiment with a similar ochre sample also from a drinking water well in Berlin.

E. coli, *E. faecalis* and *K. pneumoniae* (Figure 41, Figure 42 a, b, e) showed a log reduction of about 1 within 14 days in ochre. For *P. aeruginosa* and *L. pneumophila*, however, no or nearly no decline in colony forming units in ochre occurred within the two weeks of the experiments (Figure 41, Figure 42 c, d). Numbers of culturable *A. hydrophila* in ochre decreased about half a log unit within 14 days (Figure 41, Figure 42 f). For all target organisms the decrease of colony forming units over time of bacteria attached to ochre was less under flow-through conditions than in batch preparations in the microcosm experiments (Figure 42).

All target organisms could be detected in the water phase of the respective columns. At the beginning of the experiments numbers of target organisms in the water phase were in the range of 10^3 – 10^4 MPN or CFU/ml, which is about 0.01 – 0.2 % of the target organisms detected in the respective ochre. After 14 days those numbers were in the range of about 10^1 – 10^2 MPN or CFU/ml (Figure 41). These numbers are about one to two log units lower than in the experiments before (Figure 39), but by inoculating the ochre outside the columns it is absolutely clear that all target organisms in the water phase originate from the ochre.

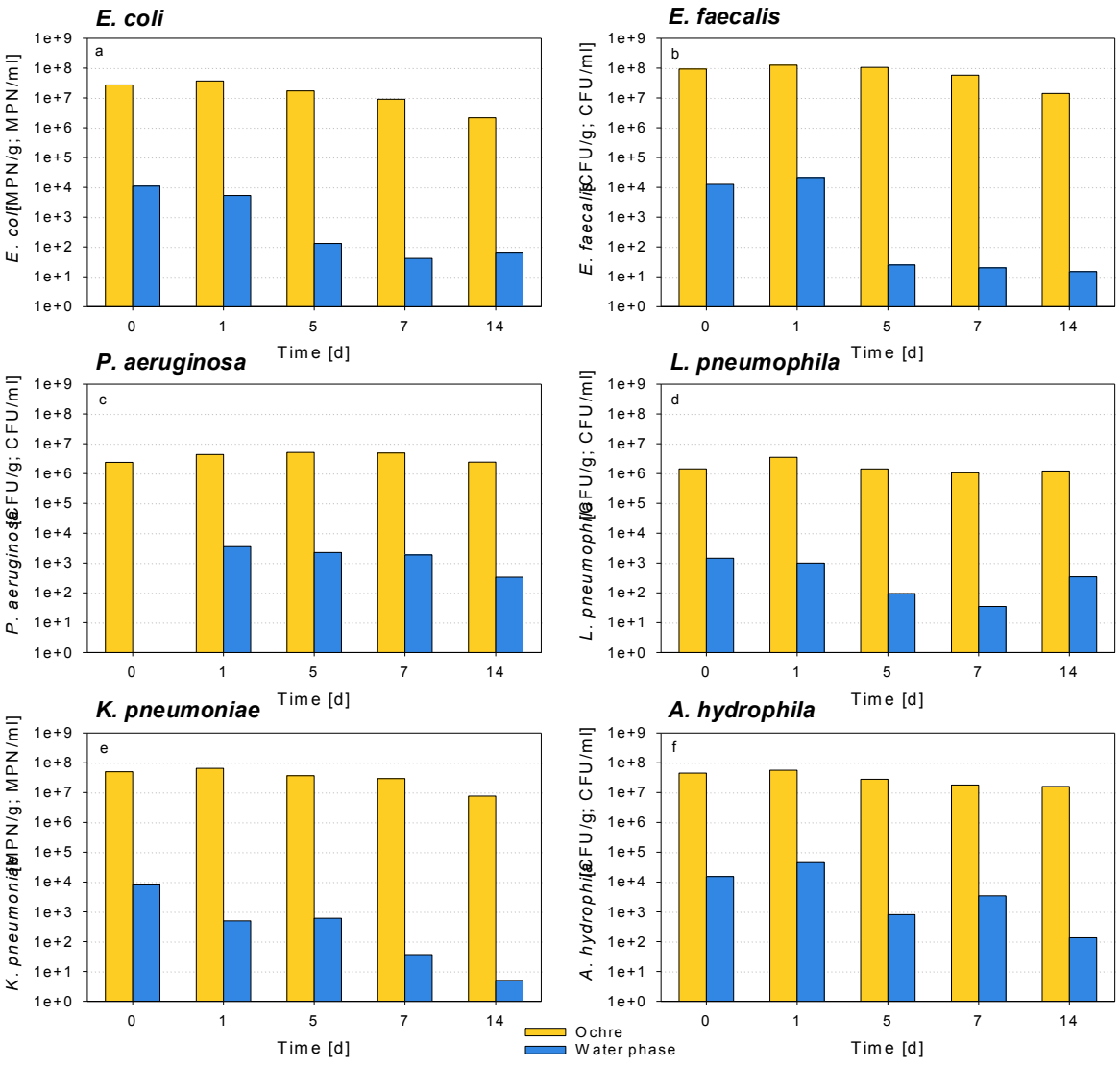

Figure 41: Survival of target organism attached to ochre (O. Tw-22) under flow-through conditions – inoculation of the ochre outside the columns.

Besides analysing the ochre and the water phase for target organisms, also swab samples from different parts of the set-up were checked. Like in the experiments before, swab samples were taken from the inner surface of the influent tube and capillary, as well as from the surface of

the column (cf. Figure 12). However, in these experimental runs, in which the ochre was inoculated outside the columns, it was not possible to have a control column without ochre but inoculated with target organisms, so columns filled with ochre in the lower half had to be sampled. It was tried only to swab the upper half of the inner surface of the column, but usually the glass surface was not completely clean from ochre particles.

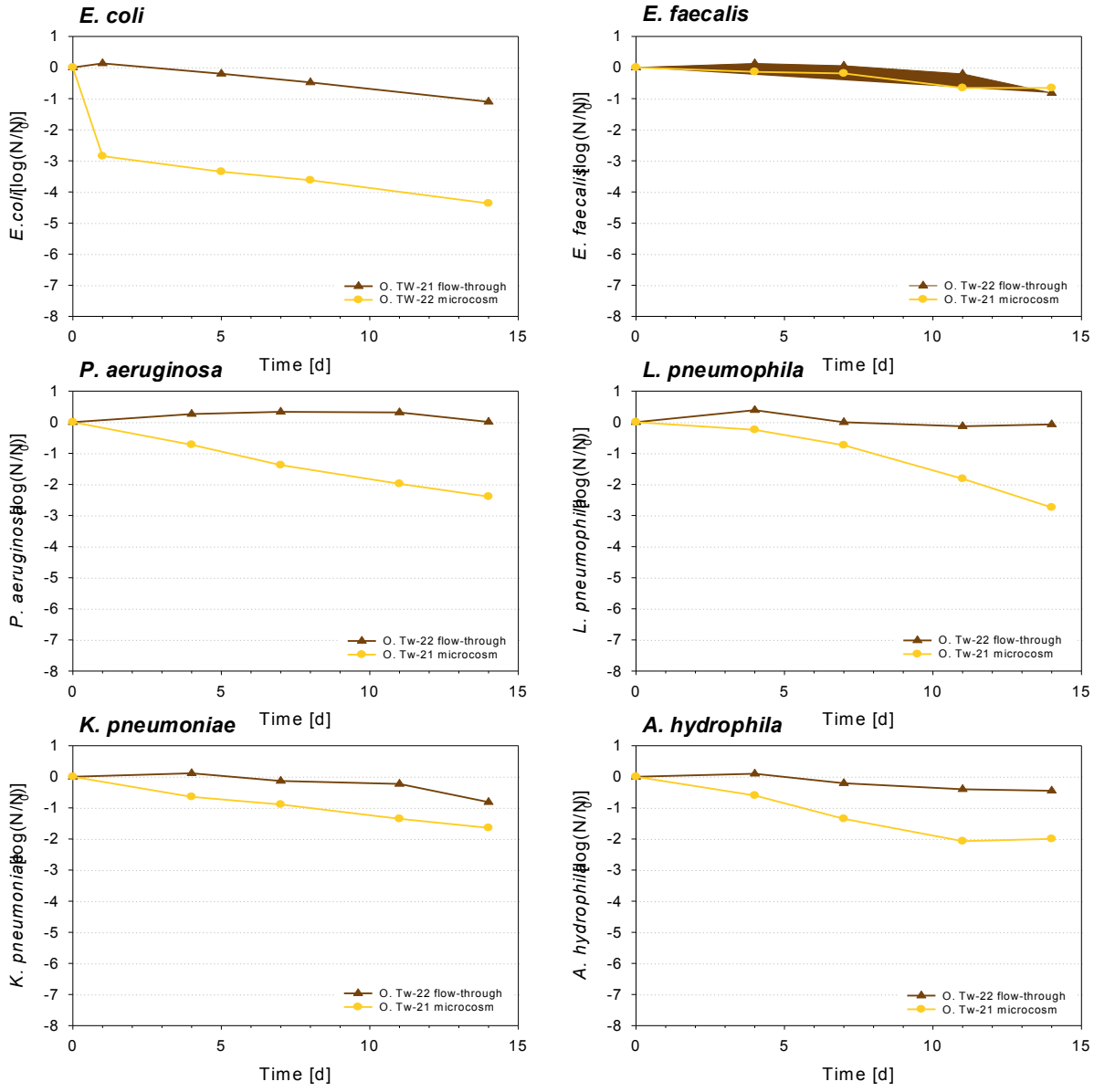

Figure 42: Survival of target organisms attached to ochre under flow-through conditions or in batch preparations. O. Tw-22 flow-through: Results from the experiments also illustrated in Figure 41, but given in $\log(N/N_0)$. N: Concentration [MPN or CFU/g ochre wet mass] at the respective sampling day; N_0: Concentration [MPN or CFU/g ochre wet mass] at day 0. O. Tw-21 microcosm: For comparison; results from run I of the microcosm experiment using ochre Tw-21 in batch preparations (compare section 3.5.3).

Also the synthetic groundwater in the storage vessel was tested for target organisms again. Results are shown in Figure 43.

Just in one single case, at day 7 of the experimental run testing the survival of *P. aeruginosa*, target organisms were detected in another part of the experimental set-up than the column. *P. aeruginosa* was cultivated from the swab sample of the influent tube and capillary in numbers of 3.0 x 10^3 and 6.0 x 10^3 CFU/sample, respectively.

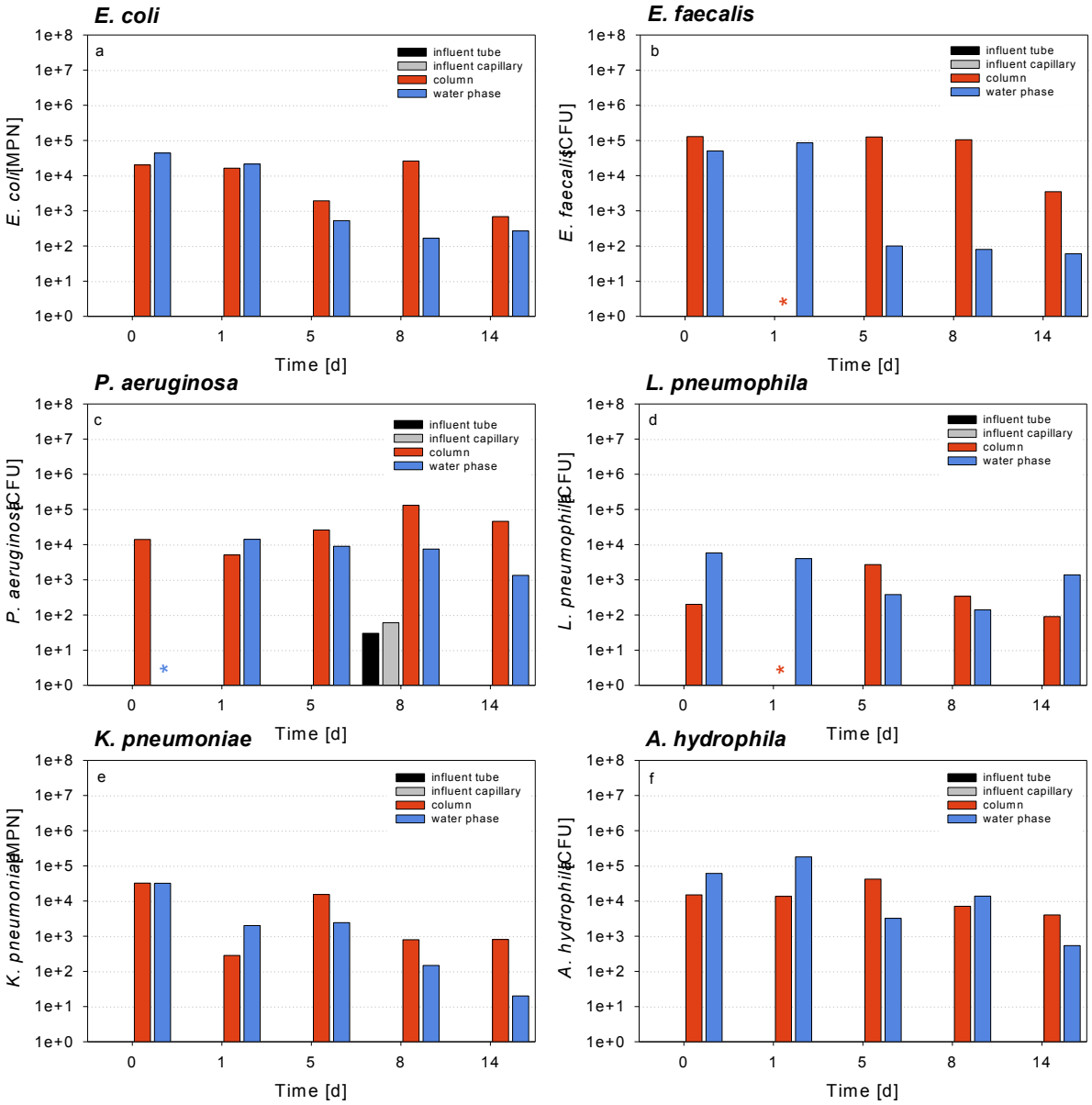

Figure 43: Test for target organisms in different parts of the experimental setup – columns half-filled with ochre (O. Tw-22) spiked with target organisms. Analysed were swap samples from the inner surface of the influent tube and capillary and of the upper half of the respective column, filled with ochre in the lower half; the water phase from the columns and the synthetic groundwater (sGW) in the storage vessel were also tested (cf. Figure 12). No target organisms were detected in the samples from the storage vessel. Numbers of target organisms given in the diagrams refer to the respective total surface or total volume sampled. *No sample; *< 5.0 x 10^2.

Numbers of target organisms on the surface of the column were in the range of 10^2 – 10^5 MPN or CFU/sample (Figure 43); that is about two orders of magnitude lower than in the experiments before (Figure 40). The whole water phase contained about 10^1 – 10^5 MPN or

CFU/sample (Figure 43), which is about one to three log units less than in the other experiments (Figure 40). All these target organisms originate from the ochre. No target organisms were cultivated from the synthetic groundwater samples from the storage vessel.

3.7 Summary survival of hygienically relevant bacteria in ochre and well water

Table 29 gives the log reduction of the different bacteria after 14 days in ochre and water samples, to summarise the results of the survival experiments with faecal indicator organisms and opportunistic pathogens in ochre suspension or in well water in microcosms, and attached to ochre under flow-through conditions. In addition, the days for 1 log reduction and the log reduction per hour at day 14 are given, to allow a better comparison with literature data.

Table 29: Overview survival of hygienically relevant bacteria in ochre or well water after 14 days. Results of the microcosm and flow-through experiments given as log(N/N_0) at day 14 and days for 1 log reduction. N: Concentration [MPN or CFU/ml] at the respective sampling day; N_0: Concentration [MPN or CFU/ml] at day 0. *E.c., E. coli; E. f., E. faecalis; P.a., P. aeruginosa; L.p., L. pneumophila; K.p., K. pneumoniae; A.h., A. hydrophila.* -, not detected/determined. [(a)] detected only on days 4, 6 and 11; [(b)] -2.5 at day 6; [(c)] -2.5 at day 11; [(d)] -3.9 at day 7; [(e)] -3.5 at day 7; [(f)] not detected after day 0; [(g)] detected in run I at day 0, in run II at all days but day0. n.r., no reduction. sd, standard deviation.

Ochre			**Log(N/N_0) at day 14**			
Microcosm	***E. c.***	***E. f.***	***P. a.***	***L. p.***	***K. p.***	***A. h.***
BWB	-1.5	-3.1	-0.4	0.5	-2.0	- [(d)]
HS 1362	-1.8	-4.6	-4.3	0.8	-1.0	- [(e)]
VK 24	-1.4	0.1	- [(a)]	- [(b)]	-0.9	- [(f)]
WR 2378	-3.5	-1.6	-3.9	- [(c)]	-0.7	-3.7
H 1424	-2.0	-0.4	-0.5	-1.2	-2.0	-1.5
W 5465	-2.5	0.6	-1.7	-0.7	-2.5	-1.7
NW 83	-1.5	-0.1	-3.2	-0.3	-1.2	-3.1
Tw-21	-4.0	-0.7	-2.3	-2.2	-1.6	-1.7
To-05	-4.6	-1.7	-2.1	-0.7	-1.1	-1.8
FRI-25	-4.0	0.0	-1.5	-1.8	-1.2	-1.7
Mean	**-2.7**	**-1.2**	**-2.2**	**-0.7**	**-1.4**	**-2.2**
Sd	1.1	1.5	1.3	1.0	0.5	0.8
Flow-through						
Tw-22	**-1.1**	**-0.8**	**0.0**	**-0.1**	**-0.8**	**-0.4**
Water			**Log(N/N_0) at day 14**			
Microcosm	***E. c.***	***E. f.***	***P. a.***	***L. p.***	***K. p.***	***A. h.***
H 1424	-2.9	-3.2	-1.9	-0.9	-3.6	-2.0
W 5465	-3.7	-1.2	-2.8	-0.4	-3.8	-2.8
NW 83	-4.7	-2.0	-2.4	-2.3	-5.0	- [(g)]
Tw-21	-1.0	-0.5	-0.6	-0.7	-0.7	-1.5
To-05	-2.3	-0.9	-1.0	-0.4	-0.7	-1.9
FRI-25	-1.5	-0.2	-0.3	-0.2	-1.8	-1.9
Mean	**-2.7**	**-1.3**	**-1.5**	**-0.8**	**-2.6**	**-2.0**
Sd	1.2	1.0	0.9	0.7	1.6	0.4

Ochre			**Days for 1 log reduction (T_{90})**			
Microcosm	***E. c.***	***E. f.***	***P. a.***	***L. p.***	***K. p.***	***A. h.***
BWB	9	5	37	n.r.	7	-
HS 1362	8	3	3	n.r.	14	-
VK 24	10	n.r.	-	-	16	-
WR 2378	4	9	4	-	19	4
H 1424	7	33	29	11	7	9
W 5465	6	n.r.	8	20	6	8
NW 83	9	140	4	42	12	5
Tw-21	3	19	6	6	9	8
To-05	3	8	7	20	13	8
FRI-25	4	n.r.	9	8	12	8
Mean	**6**	**31**	**12**	**18**	**11**	**7**
Sd	3	46	12	12	4	2
Flow-through						
Tw-22	**13**	**18**	**n.r.**	**140**	**18**	**35**
Water			**Days for 1 log reduction (T_{90})**			
Microcosm	***E. c.***	***E. f.***	***P. a.***	***L. p.***	***K. p.***	***A. h.***
H 1424	5	4	7	16	4	7
W 5465	4	11	5	40	4	5
NW 83	3	7	6	6	3	-
TW-21	14	29	22	22	21	9
TO-05	6	16	15	33	19	8
FRI-25	9	93	48	82	8	7
Mean	**7**	**27**	**17**	**33**	**10**	**7**
Sd	4	31	15	25	8	1

Overall, *E. coli* showed the most pronounced and *L. pneumophila* the least decrease in colony forming units over the course of time, with a log reduction of about 3 and about 1 after 14 days, respectively, both in ochre suspension and in well water microcosms. Considering all target bacteria in total, a similar proportion of the population survived in a culturable state in microcosms containing ochre suspension compared to those containing well water, whereas under flow-through conditions attached to ochre the proportion which survived was even higher. In the latter case, a mean log reduction of all target bacteria of about 0.5 at day 14 was observed, compared to mean log reductions of 1.7 and 1.8 in ochre suspension and well water, respectively. This corresponds to about 13 to 140 days for 1 log reduction for *E. coli* and *L. pneumophila*, respectively, under flow-through conditions attached to ochre. For *P. aeruginosa* no time span for a 1 log reduction could be calculated, because over the course of 14 days no reduction in colony forming units under flow-through conditions attached to ochre was detected.

3.8 Disinfection experiments with H_2O_2

A disinfection of a well might be necessary in case the bacteriological examination of the raw water, after a well rehabilitation, yields a result exceeding the limit values of the German Drinking Water Ordinance (ANONYMOUS 2007). For that purpose, normally, hydrogen peroxide is suggested with an application concentration of about 150 mg/l (ANONYMOUS 2001b). On the other hand, the German work sheet on well rehabilitation (DVGW W 130, ANONYMOUS 2007) recommends a periodic disinfection of wells, e.g. a monthly treatment with hydrogen peroxide with an application concentration of about 150 mg/l, to decelerate the biotic formation of ochreous deposits in wells. Such a periodic disinfection, pre-emptively applied in order to decelerate well ageing, is used, for instance, by the Berliner Wasserbetriebe at their wells. During such a treatment procedure, a hydrogen peroxide solution of 1-2 % is injected into the well, yielding a target concentration of about 300 ppm, if evenly distributed in the whole water column of the well, or locally a much higher concentration of about 10,000 ppm, if only added at one spot (just above the upper edge of the filter; H. Schwarzmüller, KWB, KompetenzZentrum Wasser Berlin, person. comm.).

Highlighted by this background, above mentioned hydrogen peroxide concentrations, 0.15 g/l, 0.3 g/l and 10 g/l, and also further concentrations (1 g/l; 1 %, 2 % and 3 %, corresponding to about 12, 23 and 34 g/l) were used to treat ochre with *E. coli*, or *E. faecalis* or *P. aeruginosa* attached to it. These conditions simulate the case that an ochre-incrusted well, contaminated by hygienically relevant bacteria, which could not be cleaned completely during well rehabilitation (cf. section 1.7) and, therefore, still contains contaminated ochre, is treated with hydrogen peroxide. To characterise the treatment conditions, the macroscopic effect of the reaction of hydrogen peroxide and ochre was observed and H_2O_2 concentration over time in contact with ochre suspension or synthetic groundwater both containing target bacteria was analysed. The effect of hydrogen peroxide on the survival of the target bacteria attached to ochre was studied using cultivation methods.

3.8.1 Reactions of hydrogen peroxide and ochre – macroscopic observations

Ochre spiked with either *E. coli*, or *E. faecalis* or *P. aeruginosa* was treated with H_2O_2 in different concentrations and using different experimental procedures.

Figure 44: Disinfection experiments using H_2O_2 in different concentrations under stagnant or flow-through conditions against hygienically relevant bacteria attached to ochre (O. Tw-22). (a) Stagnant conditions in columns, top-down: synthetic groundwater (control), H_2O_2 0.3 g/l, H_2O_2 1 g/l; Flow-through conditions, (b) H_2O_2 10 g/l, (c) Experimental set-up, columns from this experiment: Ochre spiked with (d) *E. coli* or (e) *E. faecalis*, top-down: synthetic groundwater (control), H_2O_2, 12 g/l, H_2O_2, 23 g/l and H_2O_2, 34 g/l.

Performing H_2O_2 treatment in columns resulted in the following observations: Directly after injection of H_2O_2 into the columns a development of gas could be observed. The vehemence of the reaction varied depending on the concentration of H_2O_2 and if the solution was added once or continuously. A H_2O_2 solution of a concentration of 0.3 g/l added once into the column (stagnant conditions) led to the formation of small gas bubbles while the ochre was loosened at the surface (Figure 44 a, column in the middle). The one-time application of H_2O_2

(1 g/l) resulted in big gas bubbles, fissures in the ochre layer, and the surface of the ochre was split into flakes (Figure 44 a , lowermost column).

The continuous addition of 0.3 g/l H_2O_2 caused large gas bubbles and fissures in the ochre layer. 1 g/l H_2O_2 flowing-through the column yielded, however, very big gas bubbles which displaced nearly the whole water phase while splits through the whole ochre layer could be observed. Pumping of 10 g/l H_2O_2 solution through a column half-filled with ochre resulted in the formation of very big gas bubbles, which pushed out the water phase. Gas formation continued, and the water phase was turbid and ochre coloured. Discharge of ochre from the column (Figure 44 b, c). The ochre was completely fissured, and the fissures contained gas bubbles.

3.8.2 Hydrogen peroxide concentrations in the experimental preparations

As it is known, on the one hand, that H_2O_2 rapidly decomposes in presence of granular goethite (α-FeOOH) particles (LIN & GUROL 1998) and, on the other hand, that virtually all organisms maintain high titers of enzymes that scavenge hydrogen peroxide (IMLAY 2008), such as catalase, H_2O_2 concentrations were checked in the different disinfection experiments. H_2O_2 solutions mixed with ochre suspensions or synthetic groundwater samples each containing either *E. coli*, or *E. faecalis* or *P. aeruginosa* were tested for their H_2O_2 content by permanganometric titration after different periods of time.

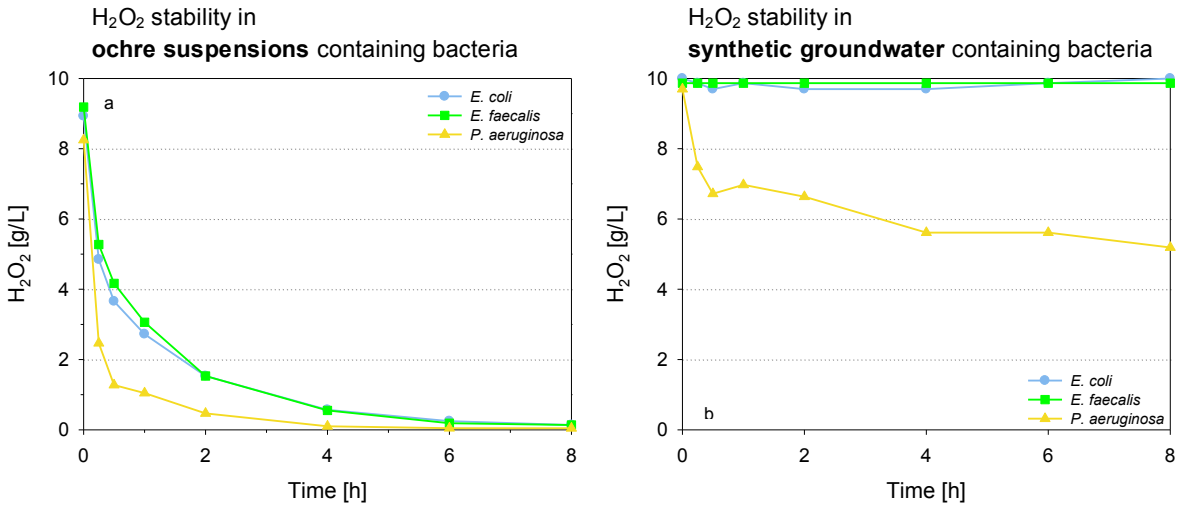

Figure 45: H_2O_2 stability over time. A hydrogen peroxide solution was mixed with ochre (Tw-22) suspensions (0.03 g dry mass/ml) or synthetic groundwater samples each containing either *E. coli*, or *E. faecalis* or *P. aeruginosa* (1 x 10^8 cells/ml). Initial H_2O_2 concentration of the preparations: 10 g/l. Method to determine H_2O_2 concentrations: Permanganometric titration.

In the different ochre suspensions the hydrogen peroxide concentration rapidly declined over time (Figure 45 a). The decline was similar in the suspensions containing *E. coli* or *E. faecalis*. But in the ochre suspension containing *P. aeruginosa*, the H_2O_2 concentration even decreased more rapidly over time. After 15 minutes the ochre suspensions contained only about 50 % of the initial H_2O_2 concentration in the preparations either with *E. coli* or *E. faecalis*, whereas the one with *P. aeruginosa* contained just about 25 % of the initial H_2O_2 concentration. After two hours a decrease in H_2O_2 concentrations of about 85 % or 95 % of the initial 10 g/l was detected in the ochre suspension with *E. coli* or *E. faecalis* and *P. aeruginosa*, respectively.

In synthetic groundwater (Figure 45 b) containing either *E. coli* or *E. faecalis*, on the other hand, no decline in H_2O_2 concentration over time was observed. In contrast, mixing hydrogen peroxide solution with synthetic groundwater containing *P. aeruginosa* resulted in a decrease in H_2O_2 concentrations over time, as well, but a considerably less pronounced one than in the ochre suspensions. After 15 minutes the mixture contained about 75 % of the initial H_2O_2 concentration and after 8 hours about half of the initial 10 g/l. Clearly, ochre is catalysing the decay of hydrogen peroxide.

3.8.3 Effect of hydrogen peroxide on the survival of hygienically relevant bacteria attached to ochre

In concentrations of up to 1 g/l hydrogen peroxide had nearly no effect on the survival of either *E. coli* or *E. faecalis* attached to ochre (Tw-22); even if the H_2O_2 solution was added continuously over 24 hours to the ochre samples spiked with the target organism (Figure 46 a).

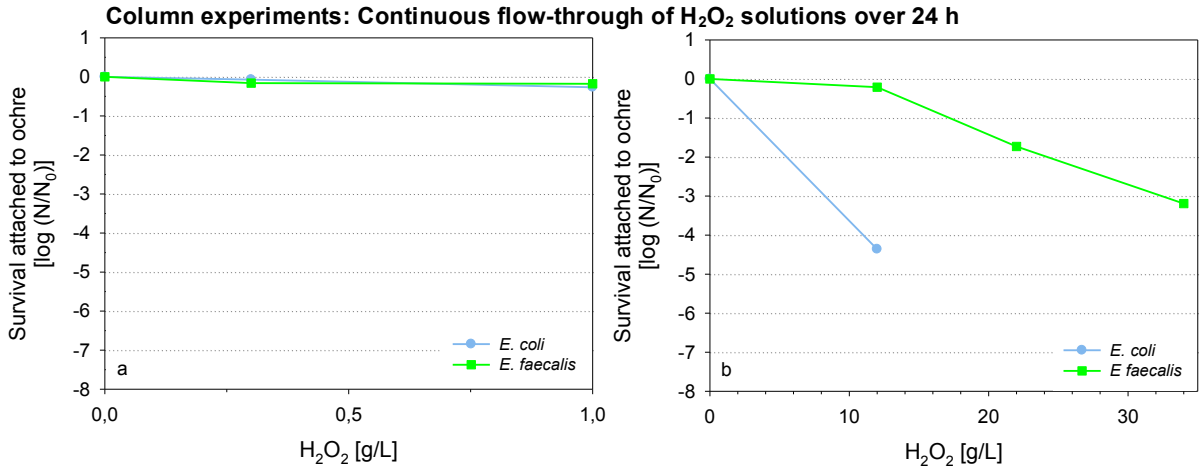

Figure 46: Survival of *E. coli* or *E. faecalis* attached to ochre (Tw-22) after treatment with H_2O_2 solutions of different concentrations for 24 h in columns under continuous flow-through of the H_2O_2 solutions. N: Concentration [CFU/g ochre wet mass] at the respective H_2O_2 concentration; N_0: Concentration [CFU/g ochre wet mass] at a H_2O_2 concentration of 0 g/l.

Only in concentrations of more than 10 g/l H_2O_2, if added continuously over 24 h, numbers of colony forming units of the faecal indicator bacteria decreased (Figure 46 b). Numbers of culturable *E. coli* attached to ochre dropped for 4.4 log units, when treated with a hydrogen peroxide solution of 12 g/l H_2O_2 continuously over 24 h. *E. faecalis*, however, still only decreased about 0.2 log units when treated with 12 g/l H_2O_2 continuously over 24 h. At higher concentrations of 22 g/l or 34 g/l H_2O_2 *E. coli* was no longer detected by cultivation and culturable *E. faecalis* declined by 1.7 and 3.2 log units, respectively, when hydrogen peroxide solutions were continuously pumped through the columns containing the spiked ochre sample.

However, in treatment of wells with hydrogen peroxide, the H_2O_2 solution is not added continuously but only once, and then left stagnant over 24 hours. In order to simulate this, disinfection efficacy against *E. coli*, *E. faecalis* and *P. aeruginosa* attached to ochre (Tw-22) was tested by adding hydrogen peroxide solutions of different concentrations once to the ochre sample spiked with the different hygienically relevant bacteria, and then leaving the preparations stagnant for 24 h. Such H_2O_2 treatment, however, only resulted in a minor decrease of colony forming units of the different bacteria (Figure 47). Numbers of culturable *E. coli*, *E. faecalis* and *P. aeruginosa* attached to ochre only declined 0.9, 0.4 and 0.5 log units at most, respectively, even if treated with hydrogen peroxide solutions of as much as 34 g/l H_2O_2.

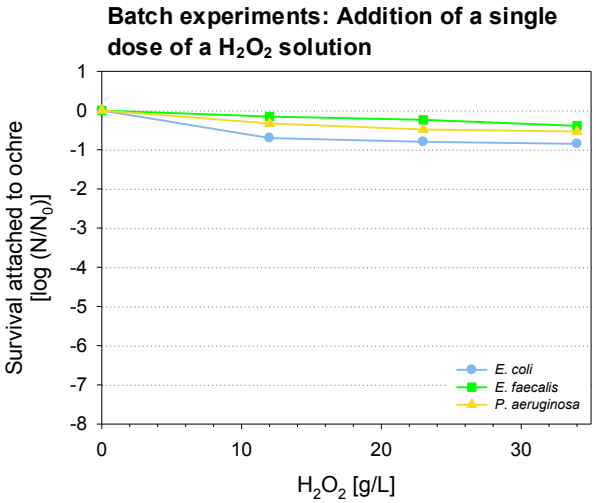

Figure 47: Survival of *E. coli*, *E. faecalis* or *P. aeruginosa* attached to ochre (Tw-22) after treatment with H_2O_2 solutions of different concentrations for 24 h in centrifuge tubes under stagnant conditions. N: Concentration [CFU/g ochre wet mass] at the respective H_2O_2 concentration; N_0: Concentration [CFU/g ochre wet mass] at a H_2O_2 concentration of 0 g/l.

These experiments clearly show that the hydrogen peroxide concentration of about 150 mg/l, recommended for well disinfection, and even a more than 200 times higher concentration (about 34 g/l H_2O_2), if it is just added once and left stagnant for 24 h, is ineffective against hygienically relevant bacteria attached to ochre. In the presence of ochre, hydrogen peroxide was rapidly decomposed, and also the presence of bacteria (*P. aeruginosa*) caused a decrease of the H_2O_2 concentration; most probably by producing high amounts of hydrogen peroxide scavenging enzymes. Thus, if a well has been contaminated by ingress of microbially polluted water and ochre incrustations present in such a well cannot be removed completely during well rehabilitation, an eradication of faecally derived bacteria and pathogens, attached to ochre, does not seem to be achievable just by the addition of a hydrogen peroxide solution (up to 3 % (v/v) application concentration) into the well.

4 Discussion

4.1 Background of the study

The formation of iron oxide incrustations in wells yields large, porous surfaces, which might be colonised by hygienically relevant microorganisms, if these organisms get into the incrusted wells. Such an intrusion can happen either by means of contaminated groundwater or by direct ingress into the wells. Even though, groundwater is often shielded from the immediate influence of microbial contamination by the overlying soil and unsaturated zones (PEDLEY et al. 2006), such contamination has been reported by a number of studies (MACLER & MERKLE 2000, HRUDEY et al. 2003, GALLAY et al. 2006, PEDLEY et al. 2006, SAPKOTA et al. 2007, KVITSAND & FIKSDAL 2010, MCKAY 2011, PAYMENT & LOCAS 2011, HYNDS et al. 2014). For instance, in the US up to half of the drinking water wells tested had evidence of faecal contamination (MACLER & MERKLE 2000), and an investigation of groundwater contamination in Canada and the US showed that about 15 % of the groundwater samples were positive for enteric pathogens (HYNDS et al. 2014). Three potential contamination pathways were identified in this study:

1. Precipitation patterns (leading to contamination ingress at the wellhead or rapid subsurface infiltration)
2. Geological pathways (increased contaminant transmissivity via unconsolidated/ fractured aquifer materials or increased runoff coefficients)
3. Inadequate source design/construction (e.g. uncovered wellhead, cracked jointing, absence of sanitary seal etc.) (HYNDS et al. 2014)

The finding that extreme precipitation plays a major role in waterborne disease outbreaks (CURRIERO et al. 2001) is of special concern because the frequency of heavy precipitation and flooding events is likely to increase due to climate change, leading to a greater contamination of both surface and groundwater (COFFEY et al. 2014, ASHBOLT 2010).

The presence of pathogens in groundwater, however, can pose a significant threat to public health, since many groundwater sources are used for public supply with a minimum level of treatment, normally chlorination, or with no treatment at all (PEDLEY et al. 2006). Therefore, in Sweden, Finland and the US, for instance, groundwater accounts for more than half of the reported waterborne outbreaks (KVITSAND & FIKSDAL 2010). The predominant recognised illness resulting from infection with pathogens in groundwater is acute gastrointestinal illness, which is mostly self-resolving in otherwise healthy people, but may be chronic, severe, or

fatal in susceptible people such as elderly, infants, pregnant women, and especially the immune-suppressed and immuno-compromised (MACLER & MERKLE 2000).

In this context, incrustations in wells could be of significance, if they support the survival of allochthonous, pathogenic microorganisms in wells, and function as a reservoir for these organisms, from which they are again released into the water. Attachment of bacteria to surfaces, that is to say the biofilm mode of life, has several ecological advantages, especially in oligotrophic environments. These include, amongst others, the protection against biocides and other stresses, nutrient availability in the form of e.g. particulate biodegradable matter or lysed cells, and the retention of extracellular enzymes in the biofilm matrix, generating a versatile external digestive system (FLEMMING 2008, FLEMMING & WINGENDER 2010). Consequently, biofilms develop on all surfaces in contact with non-sterile water, for instance, in water storage tanks, drinking-water distribution systems and domestic plumbing systems, and it has become obvious that microorganisms with pathogenic properties can persist and multiply in biofilms of man-made water systems (WINGENDER 2011, WINGENDER & FLEMMING 2011). Though, not only surfaces of water tanks and pipes are colonised by microorganisms, but also soft pipeline deposits, mainly composed of iron, manganese, aluminium and calcium, are key sites for microbial growth in drinking water distribution systems (GAUTHIER et al. 1999, ZACHEUS et al. 2001, LEHTOLA et al. 2004). Also coliform bacteria were often isolated from those deposit samples (ZACHEUS et al. 2001). Thus, incrustations and deposits in water wells may also provide micro-habitats for hygienically relevant microorganisms and pathogens, where they may survive and persist, even in the presence of a disinfectant, and are then a potential source of perseverative water contamination.

Therefore, the survival of faecal indicator bacteria and opportunistic pathogens in ochre suspension in microcosms, or attached to ochre under flow-through conditions was investigated, as well as the release of these bacteria from the ochre into the water phase, and the effectiveness of hydrogen peroxide treatment against hygienically relevant bacteria attached to ochre.

4.2 Sampling, samples and detection methods for target organisms

4.2.1 Ochre sampling

Ochre samples could be obtained from both dewatering and drinking water production wells. In general, the former contained higher amounts of ochreous incrustations than the latter and ochre could be gained nearly from each dewatering well sampled. From the drinking water

wells, on the other hand, only in about half of the cases an ochre sample could be taken, whereas the pumps from the other drinking water wells were clean, with no visible ochreous incrustations attached to it.

The finding that wells subjected to dewatering processes are especially prone to clogging by the formation of incrustations is also described in literature (e.g. LARROQUE & FRANCESCHI 2011, WEIDNER et al. 2011, WANG et al. 2014). Dewatering of aquifers, in well operation in open pit mining, leads to a strong drawdown and low operating water level in the wells, so that oxygen inside the well tube can reach reduced groundwater. Oxygen entering the groundwater may favour both chemical and biological iron oxidation. Encrustation of pumps leads to malfunction and a reduced pump life, which can be less than two months in dewatering wells (WANG et al. 2014). The formation of precipitates in such wells, thus, results in the necessity of frequent well regeneration measures, which are expensive and time consuming, especially considering that in open pit mining areas large numbers of wells are in operation, e.g. about 1500 wells in the surface mining area Garzweiler, Hambach and Inden in North-Rhine Westphalia.

In drinking water wells the formation of ochre proceeds more slowly, but also these wells can show significant incrustations after only 1 ½ years operation time (KREMS 1972).

4.2.2 Composition of the ochre

As expected, the elemental analysis of the ochre samples showed that iron is the dominating element. This is in accordance with results given by HOUBEN (2003a). Analysing incrustation samples from 70 well fields all over Germany, he found that oxides of Fe(III) represent the most common incrustation type. On the other hand, HOUBEN points out that iron oxides can take up significant concentrations of anions and cations due to their large surface area and high surface-charge density. Hydroxyl groups can be replaced by sorption of anionic ligands; the sorption affinity is especially high for small and highly charged anions such as phosphate. Cation sorption occurs via deprotonation of surface hydroxyl groups, and some anions can promote the adsorption of cations. Moreover, metal ions of similar ionic radius (such as Mn^{III}, Co^{III}, Ni^{II}, Cu^{II} and Zn^{II}) can also be incorporated directly into the crystal structure of Fe oxides via isomorphous substitution (HOUBEN 2003a). Therefore, iron precipitates from wells may contain elevated concentrations of heavy metals.

However, the ochre samples analysed in the present study overall did not contain high amounts of heavy metals. Lead, cadmium and chromium contents were below the detection

limit in most samples. Only nickel was detected in all ochre samples, but just in one sample, O. HS 1362, with a mass fraction (Ni: 0.293 g/kg) similar to the example given by HOUBEN (2003a; Ni: 0.3 g/kg). Ochre HS 1362 also contained a high content of copper, 3.98 g/kg, which is significantly higher than the values given by WALTER (1997) for screen-encrusting material from wells in Suffolk County, N.Y. (< 0.2 – 1 g/kg).

These differences, and also the differences in the elemental composition between the ochre samples in the present work, may be on the one hand due to the composition of the respective groundwater, its salt content and also its pH, and on the other hand dependent on the age of the precipitates. The chemical composition of groundwater, in turn, results from the chemical interaction between the pore water and the solid matrix through which the pore water flows, and is also affected by human impacts, such as land use and related compound intakes (WENDLAND et al. 2008), and therefore strongly depends on the particular local conditions. Sorption of anions and cations onto ochreous deposits both are strongly dependent on pH by protonation or deprotonation of the surface hydroxyl groups with decreasing and increasing pH, respectively (GADDE & LAITINEN 1974, HOUBEN 2003a, PHUENGPRASOP et al. 2011). And also the competition between different cations or anions has to be taken into account (PHUENGPRASOP et al. 2011).

The aging of ochreous deposits, that is the re-crystallisation of ferrihydrite to goethite, involves a drastic decrease of surface area and subsequently a loss of sorption capacity (HOUBEN 2003a). The re-crystallisation triggers an expulsion of large amounts of sorbed anions, while Zn, Cu, Ni and Co increased, which is probably due to uptake into the crystal structure of goethite (HOUBEN 2003a).

The number of factors influencing the genesis, mineralogy and geochemistry of iron oxide incrustations in wells and their interactions, thus, explain the heterogeneous chemical composition of the different ochre samples.

4.2.3 Ochre dissolution experiments

An elegant strategy to quantify ochre-attached bacteria would have been the dissolution of the ochre matrix. Unfortunately, this was not successful. The heterogeneous composition of the ochre samples might explain the different and not reproducible results of the ochre dissolution experiments. HOUBEN (2003b) used only freshly synthesized Fe oxides, in order to obtain reproducible experimental conditions. He points out that the Fe oxides present in natural incrustations might behave somewhat differently, e.g. due to their trace element contents.

MEHRA & JACKSON (1960), on the other hand, used environmental samples, but they studied soil samples rich in iron oxides and not iron incrustations from wells. Furthermore, their procedure comprised heating of the dissolution mixture up to 80 °C, which was incompatible with cell quantification after iron oxide dissolution in the present study. Thus, adaptations to the methods to minimise cell damage might also have caused the inadequate dissolution of the iron oxide in the ochre samples.

4.2.4 Cultivation methods

Since for the time being, no standardised culture-independent method for the evaluation of microbial safety of drinking water is available, cultivation of indicator bacteria and other hygienically relevant bacteria remains the gold standard in routine microbiological control of drinking water. Though, cultivation has besides advantages also disadvantages or limitations, as is the case for cultivation-independent methods. Major advantages of cultivation of bacteria are that cultivation is easy to perform, standardised for a number of species(groups) and mostly inexpensive or at least less expensive than some culture-independent methods like e.g. qPCR or flow cytometry (KÖSTER et al. 2003), and thus particularly suitable for routine analysis. Also of importance is that, for instance in case of infection by a waterborne pathogen, isolation of the pathogen from clinical and environmental samples by cultivation, allows for a genetic comparison of the isolates (LÜCK 2010). But the most important advantage of cultivation also leads to its major disadvantage: Cultivation techniques always show viable, actively growing organisms whereas molecular methods may also reveal dead or inactivated target organisms or their nucleic acid (KÖSTER et al. 2003, CEUPPENS et al. 2014), what can lead to interpretation problems in relation to the associated public health risk (CEUPPENS et al. 2014). However, not all viable organisms in a sample are also culturable. Many bacteria, including a variety of important human pathogens, which normally would grow on routine bacteriological media, are known to enter a physiological state in which they are still alive, but no more culturable (OLIVER 2010). The VBNC state is considered to be a survival strategy in response to harsh environmental conditions, such as nutrient starvation, incubation outside of the normal temperature range of growth, heavy metals, or oxidative stress, from which cells, however, can exit again and regain culturability and ability to cause infection (OLIVER 2010; LI et al. 2014). Therefore, cultivation is more likely to underreport the presence of a certain target organisms than molecular methods might do (WHILEY & TAYLOR 2014), as it only detects the part of the viable population, which is also able to grow in or on a certain routine bacteriolog-

ical medium. On the other hand, molecular methods involve other difficulties and limitations, especially when allied to complex environmental samples, which are discussed in the section about FISH and qPCR 4.4.4.

4.2.4.1 Cultivation of target bacteria in presence and absence of ochre particles

All target organisms could be detected by the different cultivation techniques both in ochre suspension and well water. In general, the ochre particles seemed not to interfere with the methods, as can be deduced from similar recovery rates (ratio of CFU or MPN detected to initial cell concentration adjusted within the spiked samples) of the different target bacteria in ochre suspension as in water samples.

The good suitability of the Colilert method for samples containing suspended solids, such as various food samples, sewage sludge, faeces and soil, has been described in literature (MUIRHEAD et al. 2004). In contrast, the other MPN methods, Enterolert®-DW/Quantitray®/2000 and Pseudalert®/Quantitray®/2000, for the detection of *E. faecalis* and *P. aeruginosa*, respectively, were the only methods tested in the present study for which a high amount of ochre particles seemed to be problematic. In the first case, when 5 mg ochre dry mass/ml was present in the suspension, the method yielded results, which could not be confirmed by the spread plate method on Chromocult® Enterococci Agar, and in the second case the autofluorescence of the ochre suspension hampered signal detection at all.

The discrepancy between the results obtained by the Enterolert® and the Chromocult® Enterococci Agar technique is somewhat surprising considering that both methods are based on the determination of the activity of the same enzyme, namely β-glucosidase (MAHEUX et al. 2009). Reasons may be, on the one hand, the composition of the respective media and, on the other hand, the different cultivation techniques, liquid culture versus solid medium, providing different growth conditions. Since it has been shown before that Enterolert® underestimates the true number of enterococci and detects less enterococcal strain than Chromocult® Enterococci Agar (MAHEUX et al. 2009), the spread plate method on Chromocult® Enterococci Agar was chosen as detection method for enterococci in the present study.

Concerning the detection method for *P. aeruginosa*: The autofluorescence of ochre suspensions in the Pseudalert®/Quantitray®/2000 preparations made the signal interpretation too ambiguous, therefore, the use of Pseudomonas CN agar according to DIN EN ISO 16266 was

chosen, which is also the reference method referred to in Annex 5 Part I (c) of the German Drinking Water Ordinance (TrinkwV, ANONYMOUS 2001a).

4.2.4.2 Recovery rates of the different target bacteria

With regard to the above mentioned problem of cells being viable but nonculturable and the fact that all target bacteria of the present study are known to enter the VBNC state (OLIVER 2010), recovery rates for the different target organisms by the respective cultivation technique were defined (i.e. ratio of colony forming units to number of target cells added).

Complete recovery, and hence the best culturability, was observed for *E. coli* by Colilert®-18/Quantitray®/2000, while the spread plate method using Lactose TTC agar yielded a somewhat lower recovery rate. These findings are in accordance with those of HÖRMAN & HÄNNINEN (2006) who also observed higher *E. coli* counts detected by Colilert®-18 than by the Lactose TTC method. The authors assume that one reason for this might be due to the ability of the Colilert®-18 method to induce recovery of injured and stressed coliforms and *E. coli* in their samples, as shown in literature before (BERGER 1991, MCFETERS el al. 1995). The recovery of stressed cells should in fact increase the culturability since the VBNC state is a response to stress conditions, as described above. This might also be the reason for the good to complete recovery of *K. pneumoniae* by Colilert®-18/Quantitray®/2000 in the present work.

Another explanation might be that *E. coli* and, to a somewhat lesser extent, also *K. pneumoniae*, are just easier to cultivate than the other target organisms in this study, which showed lower mean recovery rates, namely *P. aeruginosa*, *A. hydrophila* and *L. pneumophila*. In other words, maybe the Colilert method simply matches the individual growth requirements of *E. coli* and *K. pneumoniae* better than the other cultivation methods do for the respective organism. For nonculturability of an organism maybe either the consequence of the VBNC state, which the organisms has entered, or the non-suitability of the applied cultivation conditions (AMANN et al. 1995). Therefore, the considerable proportion of nonculturable *P. aeruginosa*, *A. hydrophila* and *L. pneumophila*, could be a demonstration that these organisms more directly enter the VBNC state, than *E. coli*, *K. pneumoniae* and *E. faecalis*, or could also indicate that the media do not provide optimal growth conditions. For instance for *L. pneumophila* it has been shown that the use of selective media, like the GVPC medium, resulted in a considerable decrease in organism recovery compared to the non-selective medium BCYE (BARTIE et al. 2001). However, due to the problem of overgrowth by non-

legionellae on BCYE, the selective medium had to be chosen for the detection of *Legionella* in ochre suspensions. On the other hand, BARTIE et al. (2003) demonstrated that the culturability of legionellae in 25 % of cultures on GVPC could be improved by re-incubation of sample concentrates with autochthonous amoeba, which seem to be due to resuscitation of legionella which were in the VBNC state.

Furthermore, besides the possibility that certain growth media may have the ability to induce the recovery of stressed cells, different susceptibilities towards stress, and thus also differences in the conditions leading to an entry into the VBNC state, may explain the observed dissimilarity in recovery rates of the different target organism. The good to complete recovery of *E. faecalis* by Chromocult® Enterococci-Agar, therefore, might be due to its high intrinsic resistance towards external stresses, which enable Enterococci to grow even under hostile conditions (AUFFRAY et al. 2011).

In addition, it must also be remembered that colony forming units are only an estimate of the numbers of cells present not only because of the problem of VBNC state bacteria, but also because not every colony necessarily represents a single cell, if the cells were not well separated on the plate. A colony could arise from one cell or several thousand (SUTTON 2011). And, thus, the more firmly the cells are attached to each other, or to particles (LÜCK 2010), the less likely they will be separated to form single colonies. Low recovery rates, hence, might also be explained to some extent by insufficient separation or detachment of cells.

4.3 Hygienically relevant bacteria in contact with ochre

Allochthonous microorganisms in ochreous incrustations in wells have to cope with several different stress factors to survive in this environment. In deposits mostly composed of iron oxides, one major stress factor may be oxidative stress due to iron excess, for iron can trigger the formation of reactive oxygen species via the Fenton reaction or related reactions. Moreover, since ochreous incrustations may also contain other metals besides iron, these metals, such as copper, lead, zinc, cadmium, or others, could also affect the survival of hygienically relevant bacteria in ochre. Further parameters, which have to be considered as stressors are temperature, pH, or the indigenous microflora of the ochreous deposits and the well water.

On the other hand, ochre incrustations may represent an ecological niche for heterotrophic microorganisms by providing a large surface area for colonisation, a potential source of nutrients (in terms of organic matter contained in the ochreous deposits; cf. section 4.4.3) and pos-

sibly protection against, for instance, predation or disinfectants (cf. section 4.6) to attached bacteria.

Hence, the different effects of the diverse factors allochthonous microorganisms entering ochreous deposits in wells are confronted with could either lead to the inactivation of such microorganisms or could promote their survival. These circumstances lead to the following opposing hypotheses:

a) Hygienically relevant bacteria are inactivated in contact with ochre due to oxidative stress or other stressors.
b) Hygienically relevant bacteria integrate into the ochre matrix, survive and persist there, representing thereby also a potential source of water contamination.

These hypotheses, which were tested in survival experiments with faecal indicator bacteria and opportunistic pathogens in ochre suspensions in microcosm experiments and attached to ochre under flow-through conditions, are discussed in the following.

4.3.1 Are target bacteria inactivated due to oxidative stress?

Oxidative stress describes the cellular status when the concentration of reactive oxygen species (ROS) increases to a level that exceeds the cell's defence capacity (CABISCOL et al. 2000). In connection with iron, oxidative stress arises when ferrous (Fe^{2+}) or ferric iron (Fe^{3+}) reacts with hydrogen peroxide to produce harmful ROS, such as the highly destructive hydroxyl radical ($^{\bullet}OH$), the hydroperoxyl radical ($HO_2^{\bullet}$) or the superoxide anion radical ($O_2^{\bullet-}$) according to the following reactions (SPUHLER et al. 2010, Brassington et al. 2009, DUNFORD 2002, HENLE & LINN 1997):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad \text{(Fenton-reaction)} \quad \text{(I)}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^{\bullet} + H^+ \quad \text{(Fenton-like reaction)} \quad \text{(II)}$$

$$Fe^{3+} + H_2O_2 \rightarrow (FeOOH^{2+} + H^+) \rightarrow Fe^{2+} + 2\,H^+ + O_2^{\bullet-} \quad \text{(III)}$$

ROS cause cellular damage as a result of their high reactivity. Especially hydroxyl radicals react with whatever biomolecule is present at their site of formation (SPUHLER et al. 2010, IMLAY 2008, CABISCOL et al 2000), affecting, for instance, membranes, enzymes or DNA. Figure 48 illustrates the formation of reactive oxygen species in *E. coli*, as well as the intracellular damage caused by ROS and its repair.

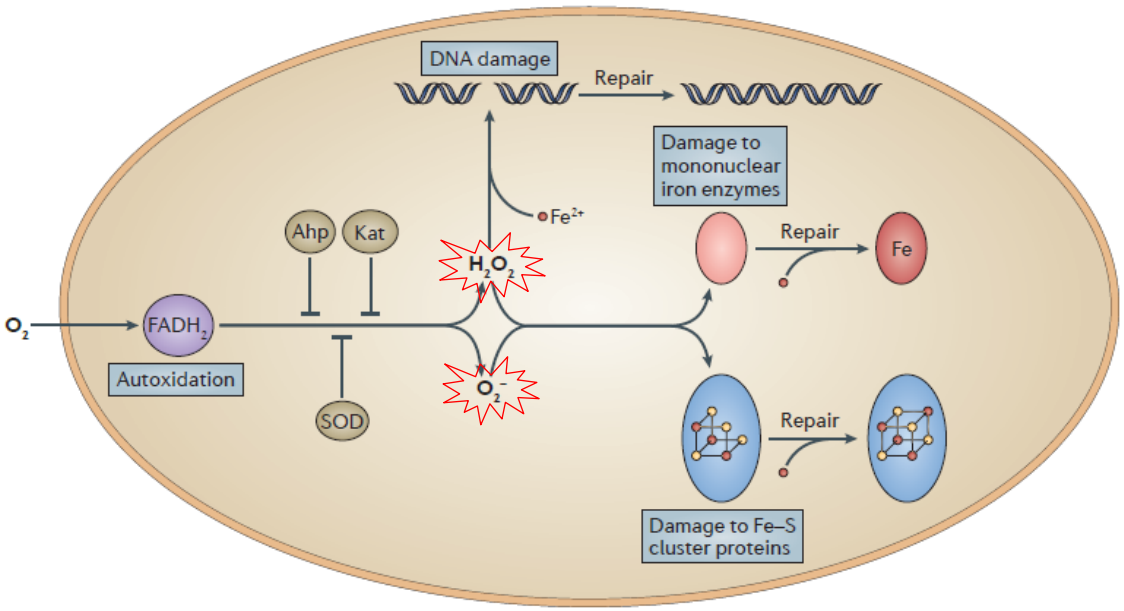

Figure 48: Reactive oxygen species in *E. coli*: Formation, damage, repair. The autoxidation of redox enzymes leads to continuous hydrogen peroxide (H_2O_2) and superoxide (O_2^-) formation. Catalases (Kat), peroxidases (e.g. alkyl hydroperoxide reductase, Ahp) and superoxide dismutases (SOD) minimize the accumulation of these two oxidants. Nevertheless, both oxidants damage mononuclear iron enzymes and Fe-S cluster proteins. The disabled enzymes are continuously repaired. H_2O_2 also reacts directly with the pool of unincorporated Fe^{2+}, which loosely associates with biomolecules, including DNA. The resultant hydroxyl radicals damage DNA, requiring the action of repair enzymes (After IMLAY 2013, modified).

For the above-mentioned reactions (I-III) to happen, there must be, on the one hand, free iron in solution and, on the other hand, there has to be a source of hydrogen peroxide.

In the ochre suspensions, free iron would exist if it is released from the iron ochre particles. A release of iron from the ochre particles should occur, since SCHULTE (2003; pp. 112-115) found free iron in the milligram per litre range leached from a corrosion product from a cast iron pipe, even though the analysed suspension contained about 100 to 250 times less ochreous solid matter (0.2 mg/ml) than the ochre suspensions in the present study (20 – 50 mg ochre dry mass/ml).

Hydrogen peroxide, on the other hand, is formed constantly inside aerobic organisms, because ROS are inevitable by-products of aerobic metabolism. A mixture of superoxide and hydrogen peroxide, i.e. partially reduced forms of oxygen, is generated inside cells when molecular oxygen abstracts electrons from exposed redox moieties of electron-transfer enzymes, in particular from flavoenzymes, which are both ubiquitous and abundant (IMLAY 2008; see Figure 48). Measurements in *E. coli* for example demonstrated that in well-fed cells H_2O_2 is formed at a constant rate of 10 – 15 µM/s (MISHRA & IMLAY 2012). On occasion bacteria may also be confronted with extra-cellular sources of hydrogen peroxide such as photochemically driven

redox reactions or chemical thiol/metal oxidations that occur at oxic-anoxic interfaces (MISHRA & IMLAY 2012; see Figure 9).

However, faecal indicator bacteria and facultative pathogens are not completely inactivated in contact with ochre. A part of the population of all target bacteria added to ochre suspensions survived over a time period of 14 days in a culturable state in the majority of ochre samples tested. A complete inactivation due to oxidative stress caused by excess iron from the ochre was not observed.

As free iron should be present in the ochre suspensions and endogenous hydrogen peroxide is constantly formed, but no complete inactivation of the hygienically relevant bacteria added to ochre takes place, the bacteria seem to cope with these stress factors. Several parameters may come into consideration for this survival of the target bacteria in the ochre suspensions, which may also play an important role for the resistance of target bacteria (*E. coli*, *E. faecalis* or *P. aeruginosa*) attached to ochre when treated with H_2O_2 as a disinfectant (cf. section 4.6):

- The tight control of uptake and storage of iron in bacteria
- The many enzymes that scavenge intracellular hydrogen peroxide
- If cellular damage by ROS has occurred, the mechanisms to repair it
- If exogenous hydrogen peroxide is formed, the possible catalytic decomposition of it on the surface of the iron oxide ochre

4.3.1.1 Control of iron uptake and storage in bacteria

Since iron is both an essential nutrient for sustaining bacterial growth, as well as a dangerous metal due to its capacity to generate ROS, iron metabolism in bacteria is strictly regulated, (TOUATI 2000, CORNELIS et al. 2011, LÓPEZ et al. 2012). Iron is not easily available for microorganisms under aerobic conditions, due to the poor solubility of its oxidised form. Therefore, bacteria produce strong extracellular Fe^{3+} chelators, termed siderophores, to mediate iron uptake (CORNELIS et al. 2011). However, as iron in its free form triggers the production of ROS, it is stored in proteins once it is taken up in the bacterial cell (CORNELIS et al. 2011). Figure 49 illustrates this uptake and storage of iron in Gram-positive and Gram-negative bacteria.

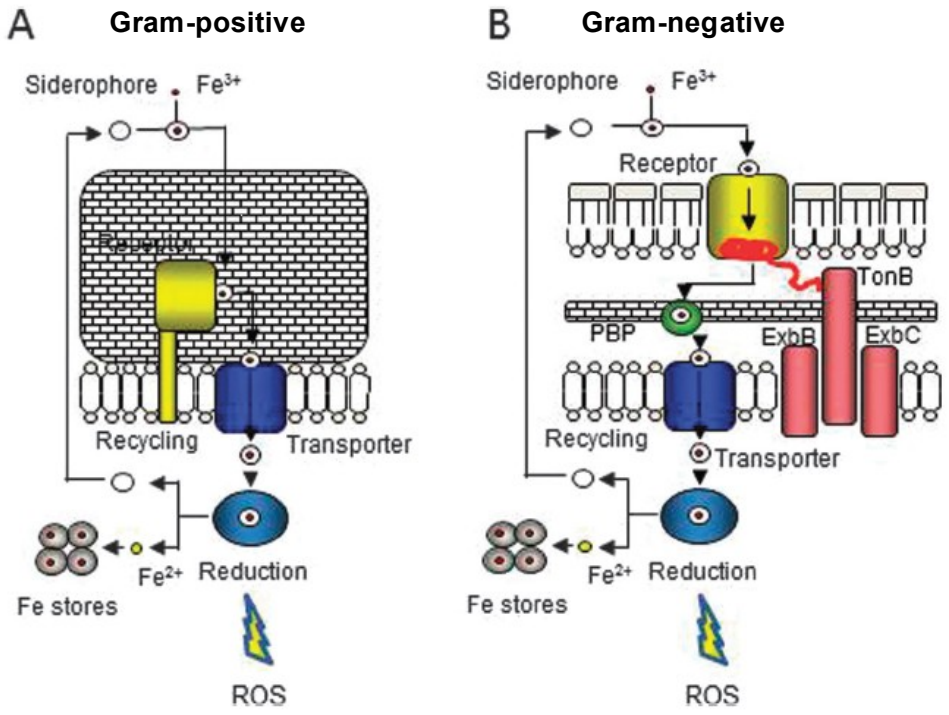

Figure 49: Siderophore-mediated iron uptake in Gram-positive (A) and in Gram-negative (B) bacteria. Fe^{3+} is chelated by a secreted siderophore and the complex binds a receptor which transfers the ferrisiderophore to a transporter either directly (in the case of Gram+ bacteria) or via a periplasmic binding protein (in the case of Gram- bacteria). Once in the cytoplasm the iron is removed by a reductive process or by the action of an esterase. Reactive oxygen species could interfere with the reductive process when the reductase contains [Fe–S] cluster(s). Fe^{2+} is stored in proteins in the bacterial cell (After CORNELIS et al. 2011, modified).

Furthermore, bacteria respond to environmental changes in iron availability by shutting down or activating the transcription of key genes encoding proteins that directly control iron metabolism (LÓPEZ et al. 2012). The most widespread regulator of iron homeostasis in bacteria appears to be Fur, the ferric uptake regulator (TOUATI 2000). Fur is a conserved protein in both Gram-positive and Gram-negative bacteria, which operates primarily as a repressor of iron uptake genes (e.g. those involved in siderophore biosynthesis or in transport across the membrane), but can also act as a positive regulator for the biosynthesis of iron storage proteins (CORNELIS et al. 2011). When cellular iron levels are high, Fur is activated by binding Fe^{2+} as a cofactor and down-regulation of iron uptake components is achieved by binding of the activated Fur to the DNA at a specific sequence, the iron box (CORNELIS et al. 2011; see Figure 10). Iron regulation, thus, enables bacteria to acquire the iron essential for survival, while maintaining low levels of free intracellular iron, which could cause oxidative stress and damage (TOUATI 2000).

4.3.1.2 ROS scavenging enzymes

Enzymes scavenging superoxide and hydrogen peroxide, primarily superoxide dismutase, peroxidases and catalases, are maintained at high titres by virtually all organisms (HENLE & LINN 1997, IMLAY 2008, MISHRA & IMLAY 2012). Besides the basal oxidative defences, which are sufficient to protect bacteria from endogenously generated oxidants, most microbes also induce additional responses when elevated levels of $O_2^{\bullet-}$ and H_2O_2 stress are artificially imposed in the laboratory (IMLAY 2008).

In *E. coli*, for example, elevated levels of H_2O_2 cause the activation of OxyR, a positive transcription factor for OxyR regulon members, such as *katG*, encoding catalase G (KatG), and *ahpCF*, encoding alkyl hydroperoxide reductase (Ahp) (IMLAY 2013; Figure 50). Ahp is a thiol-based peroxidase that transfers electrons from NADH to H_2O_2, thereby reducing it to water, and it is the primary scavenging enzyme of *E. coli* under routine growth conditions (IMLAY 2013). The synthesis of Ahp and KatG is induced more than tenfold by activated OxyR (IMLAY 2013).

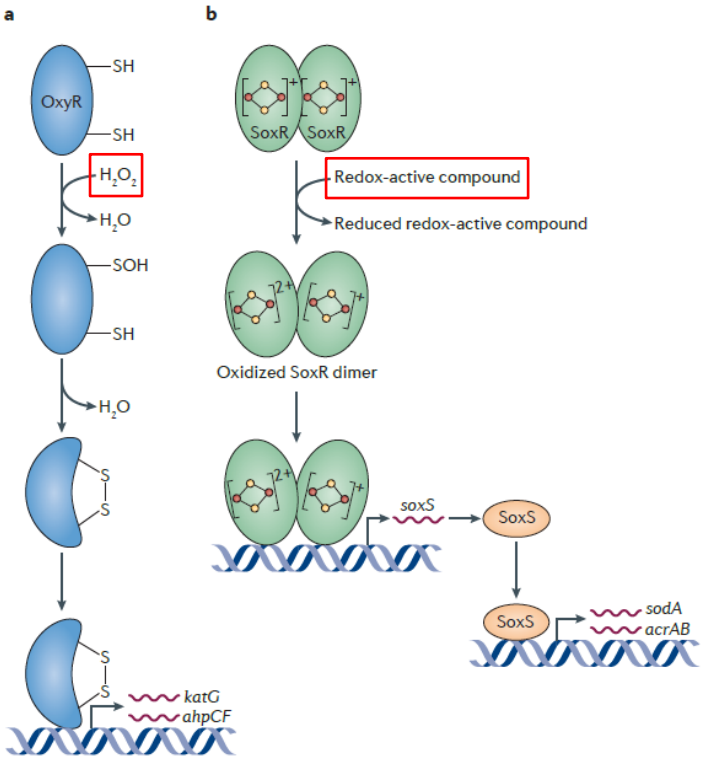

Figure 50: Defence systems in *E. coli* induced under conditions of oxidative stress. (a) OxyR system, OxyR reacts with H_2O_2 resulting in a conformation change that enables it to act as a positive transcription factor for OxyR regulon members (e.g. *katG* and *ahpCF,* encoding catalase G and alkyl hydroperoxide reductase). (b) SoxRS system, SoxR becomes activated through the oxidation by redox-active compounds (e.g. phenazines or quinones), which are produced by plants and bacterial competitors. Oxidized SoxR stimulates transcription of *soxS*, and the SoxS protein acts as a secondary transcription factor that activates the expression of SoxRS regulon members (e.g. *sodA* and *acrAB,* encoding Mn-cofactored superoxide dismutase and a multidrug efflux pump) (After Imlay 2013, modified).

In *E. faecalis*, on the other hand, the designated hydrogen peroxide regulator (HypR) has been shown to regulate the expression of alkyl hydroperoxide reductase, thiol peroxidase and glutathione peroxidase, as part of the response to hydrogen peroxide (SZEMES 2010).

By activation of redox-sensitive transcriptional regulators, under conditions of oxidative stress, concentrations of ROS scavenging enzymes can thus be adapted to the amount of oxidants present.

4.3.1.3 Repair of damage caused by ROS

ROS can damage both mononuclear iron enzymes and Fe-S cluster proteins (Figure 51), as well as DNA (see Figure 48). The disabled enzymes, however, are continuously repaired by reduction and remetallation and their steady-state activities represent the balance between damage and repair processes (IMLAY 2013; Figure 51).

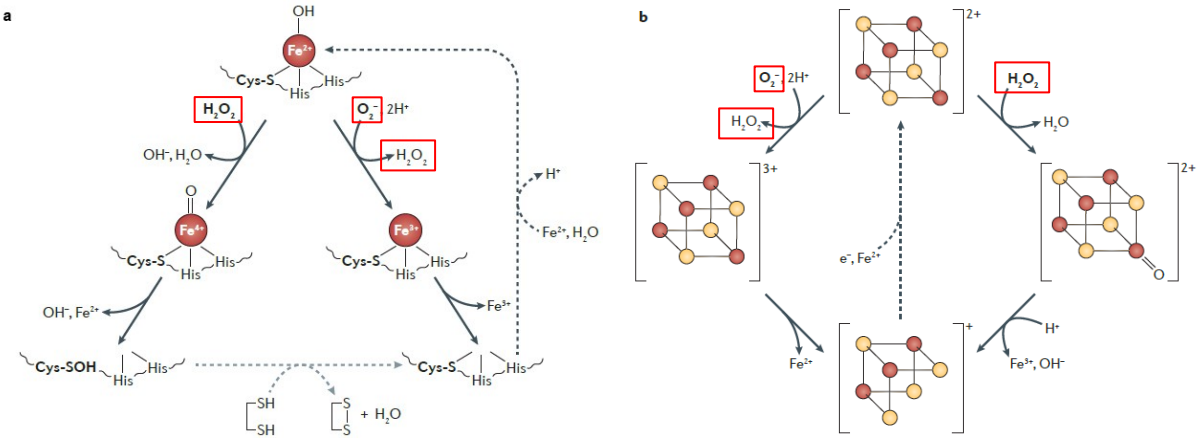

Figure 51: Oxidative vulnerability of mononuclear iron enzymes and of dehydratase [4Fe-4S] clusters. (a) Oxidation of the mononuclear iron enzyme by H_2O_2 generates a transient ferryl species (Fe^{4+}=O) that is then quenched by a coordinating Cys residue. A sulphenic species (–SOH) is the ultimate product. Reactivation requires sulphenic reduction and then remetallation (dashed grey arrows). Oxidation by O_2^- generates Fe^{3+}, which dissociates. In this case, the activity can be restored by simple remetallation (dashed black arrows). (b) Exposed cluster oxidised by superoxide (O_2^-) results in the formation of hydrogen peroxide (H_2O_2) and conversion of the cluster to an unstable $[4Fe–4S]^{3+}$ species, which then releases Fe^{2+}. The loss of the catalytic iron atom destroys enzyme activity. Oxidation of the cluster by H_2O_2 presumably creates a transient ferryl species that abstracts a second electron from the cluster; Fe^{3+} dissociates. After damage by either oxidant, the resultant $[3Fe–4S]^+$ cluster can be reactivated in vitro and in vivo by reduction and remetallation (dashed line) (After IMLAY 2013, modified).

Interestingly, besides the possibility of repairing mononuclear iron proteins, *E. coli* has an additional strategy to maintain these enzymes activity: it replaces the iron atom with manganese (IMLAY 2013). This may also play a role for its survival in iron ochre, since most ochre samples also contain certain amounts of manganese.

When DNA damage occurs, repair is essential, since a bacterial cell growing in an aerobic environment will suffer 3,000-5,000 DNA lesions per cell per generation, and most of them are oxidative in origin (RICE & COX 2001), requiring the action of repair enzymes. In *E. coli*, for example, endonucleases IV and VIII initiate the excision of oxidised bases, while exonuclease III and endonuclease IV excise fractured ribose moieties and restore a 3' primer for DNA polymerase-driven repair synthesis (IMLAY 2013).

4.3.1.4 Catalytic decomposition of hydrogen peroxide on iron oxide

Hydrogen peroxide is not only an inevitable by-product of aerobic metabolism, as described above, which is why endogenous H_2O_2 is constantly formed in bacteria, but also several exogenous sources of H_2O_2 have been identified (IMLAY 2008). For instance, hydrogen peroxide is formed by chemical processes when reduced metals and sulphur species seep from anaerobic sediments into oxygenated surface waters, or it can be produced by photochemically driven redox reactions (IMLAY 2008, MISHRA & IMLAY 2012). Furthermore, lactic bacteria excrete large doses of H_2O_2 to supress the growth of competing microbes, as well as amoeba, plants and macrophages actively generate H_2O_2 to fight invading bacteria (IMLAY 2008, MISHRA & IMLAY 2012; see Figure 9). This is why bacteria in certain occasions have to cope with natural sources of hydrogen peroxide. As an uncharged species, H_2O_2 penetrates membranes with a permeability coefficient similar to that of water and, therefore, H_2O_2 stress inside cells arises whenever hydrogen peroxide is present in their extracellular habitat (IMLAY 2008, MISHRA & IMLAY 2012). In *E. coli*, for instance, H_2O_2 influx exceeds the rate of its endogenous formation when environmental hydrogen peroxide concentrations exceed 0.2 µM (MISHRA & IMLAY 2012).

However, in environments containing heterogeneous catalysts, such as the metals Fe, Mn, Cu, Ni and their oxides, aqueous hydrogen peroxide can be rapidly decomposed (LIN & GUROL 1998). This may play a significant role in the context of oxidative stress due to exogenous hydrogen peroxide for hygienically relevant bacteria in ochre, since incrustations in wells in Germany are most commonly oxides of Fe(III) (HOUBEN 2003a), which may also contain several other metals. The decomposition of H_2O_2 on an iron oxide surface involves a series of chain reactions initiated by the formation of a precursor surface complex. A proposed reaction mechanism comprises the following reactions (LIN & GUROL 1998):

$$\equiv Fe^{III}\text{–}OH + H_2O_2 \Leftrightarrow (H_2O_2)_s \qquad (1)$$

$$(H_2O_2)_s \rightarrow \equiv Fe^{II} + H_2O + HO_2^{\bullet} \qquad (2)$$

$$\equiv Fe^{II} + H_2O_2 \rightarrow \equiv Fe^{III}–OH + {}^{\bullet}OH \quad (3)$$

$$HO_2^{\bullet} \Leftrightarrow H^+ + O_2^{\bullet-} \quad (4)$$

$$\equiv Fe^{III}–OH + HO_2^{\bullet}/O_2^{\bullet-} \rightarrow \equiv Fe^{II} + H_2O/OH^- + O_2 \quad (5)$$

$${}^{\bullet}OH + \equiv Fe^{II} \rightarrow \equiv Fe^{III}–OH \quad (6)$$

The sequence of reactions leads to the formation of different radicals, such as hydroperoxyl and hydroxyl radicals. Since these radicals are quite reactive, it is plausible that they will react with H_2O_2 and other species on the oxide surface (LIN & GUROL 1998). Therefore, bacteria in the ochre may only be harmed by radical attack if they are present at the site of the radical formation. On the other hand, the oxidative stress due to exogenous H_2O_2 will be reduced, because in contact with ochre H_2O_2 will be decomposed before it can enter bacterial cells.

Altogether, the oxidative stress encountered by faecal indicator bacteria and opportunistic pathogens in ochre suspension seems to be within the range of their adaptability considering that a part of the population of all target bacteria added to ochre suspensions survived over a time period of 14 days, in a culturable state, in the majority of ochre samples tested. This even seems to be true when hygienically relevant bacteria attached to ochre are exposed to elevated exogenous hydrogen peroxide concentrations, like in the disinfection experiments of this study, which is discussed in section 4.6.

4.4 Survival and persistence of hygienically relevant bacteria in ochre

Besides the general potential of a part of the population of faecal indicator bacteria and opportunistic pathogens to survive in ochre for at least 14 days in a culturable state, certain dissimilarity in the culturability of the target organisms in the different ochre samples over the course of time was observed. In some ochre samples the target organisms showed a more pronounced decline in colony forming units over time than in other samples. Interestingly these samples were not the same for every target organism. The reason for the different extent of inactivation of target bacteria in the different ochre samples, may be, on the one hand, the presence or absence of additional stress factors in the samples, besides the possible oxidative stress trigged by excess iron, and, on the other hand, the presence or absence of factors counteracting the inactivation and favouring the survival of the target bacteria in a culturable state.

However, the differences in culturability over time among the different target bacteria might be based on different degrees in resistance against or sensitivity towards environmental stresses, in particular if one considers that the target organisms comprise both environmental bacteria and bacteria belonging to the intestinal microflora of humans and animals.

4.4.1 Additional stressors besides iron excess and bacterial resistance mechanisms

Additional stressors could be, for instance, other metals than iron, such as copper, nickel, lead and zinc, which are toxic in excess (RADEMACHER & MASEPOHL 2012, DUPONT et al. 2011, SILVER & PHUNG 2005, JAKUBOVICS & JENKINSON 2001) or could induce the VBNC state (DWIDJOSISWOJO et al. 2011, BÉDARD et al. 2014), acidic pH (JEONG et al. 2008, VAN ELSAS et al. 2011, WANG & GU 2005), or the indigenous microbiota of the sample, attributed to competition for nutrients, antagonism or predation (BANNING et al. 2002, GORDON & TOZE 2003, VAN ELSAS et al. 2011).

4.4.1.1 Toxic metal ions

Regarding the detrimental effect of other metals than iron, ochre sample HS 1362 particularly stands out from the other samples, as it contained significantly higher amounts of especially copper, but also of chromium, lead, aluminium, nickel and zinc. The amount of copper, for instance, was 15 to 265 times higher than in the other ochre samples. Therefore, it is not surprising that both *E. faecalis* and *P. aeruginosa* showed the highest log reductions in the microcosms containing this ochre sample, and that *A. hydrophila* was even no longer detected by cultivation in this microcosm experiment after day 7. This inactivation could be explained by the toxicity of heavy metals, which is based on inducing oxidative stress and interfering with protein folding and function (TEITZEL & PARSEK 2003). Copper, even though being an essential cofactor of various enzymes, is highly toxic to living cells, when in excess; just like zinc and nickel, which are also both required as micronutrients but toxic in excess (RADEMACHER & MASEPOHL 2012, SIVER & PHUNG 2005).

Copper interacts with free proteinogenic thiol groups, destabilizes iron-sulfur cofactors, competes with other metals for protein binding sites, and possibly leads to formation of reactive oxygen species (RADEMACHER & MASEPOHL 2012). Other targets of copper toxicity, besides structural and functional proteins, are thought to include nucleic acids, as well as lipids, and inhibition of metabolic processes such as respiration and osmotic stress resulting in cell lysis (WARNES et al. 2010).

The biocidal efficacy of copper alloys against enterococci, for instance, was demonstrated by WARNES and co-workers (2010). In their study, they found a rapid die-off of pathogenic enterococci in contact to copper surfaces, with no viable cells detected on any alloys following exposure of 1 h at an inoculum concentration of ≤ 10^4 CFU/cm^2, whereas both species, *E. faecalis* and *E. faecium*, survived up to several weeks on stainless steel. This process of "contact killing" on copper surfaces appears to proceed by successive membrane damage, copper influx into the cells, oxidative damage, cell death and DNA degradation (GRASS et al. 2011). However, studies, testing the influence of copper ions on the viability of planktonic *P. aeruginosa* cells in different water samples, did not show damage to the cytoplasmic membrane, nor damage to ribosomes or decline in numbers of ribosomes, but only a loss of culturability (BÉDARD et al. 2014, DWIDJOSISWOJO et al. 2011). Exposure to 0.25 mg Cu^{2+}/l or 0.6 mg/l copper sulphate resulted in more than 6-log reduction in colony forming units, while total cell counts and concentrations of cells with intact membrane and intact 16S rRNA were constant. Quenching of the available concentration of copper ions by addition of a chelating agent resulted in recovery of culturability of copper-stressed *P. aeruginosa* within 3 days and 14 days, respectively (BÉDARD et al. 2014, DWIDJOSISWOJO et al. 2011).

In comparison, ochre microcosms in the present study, except the ones made up with ochre sample H 1424 or NW 83, contained also at least 0.6 mg Cu/l or even much higher concentrations: HS 1362 (119.4 mg/l), WR 2378 (13.0 mg/l), VK 24 (6.8 mg/l), BWB (3.0 mg/l), W 5465 (2.4 mg/l), FRI-25 (1.4 mg/l), Tw-21 and To-25 (0.6 mg/l) [calculated from the copper contents and dry residue values given in Table 23 for ochre suspensions containing 0.11 g ochre wet mass/ml]. Therefore, high amounts of copper could have contributed to high losses of culturability for *E. faecalis* in sample HS 1362, for *P. aeruginosa* in samples HS 1362, WR 2378 and VK 24, as well as for *A. hydrophila* in samples HS 1362, WR 2378, VK 24 and BWB. However, on the basis of the above findings, one could have been expected higher log reductions of culturable target bacteria in ochre microcosms than the ones found, or even complete loss of culturability. Probably this did not happen because the copper contained in the ochre samples is likely bound to the ochre matrix, and might only be partly released from it, limiting its biocidal efficacy. Besides, ochre particles contained in the preparations could also have counteracted the inactivation, as discussed below (cf. section 4.4.3, page 137f.).

Furthermore, survival of target organisms, even in a culturable state, despite the ochre samples containing considerable amounts of copper, might also be due to copper resistance and homeostasis mechanisms. Since copper is an essential trace element in most living organisms,

with more than 30 types of copper-containing proteins known today (GRASS et al. 2011), while on the other hand free copper is highly toxic, cells need to tightly control copper homeostasis to maintain metabolism and viability (RADEMACHER & MASEPOHL 2012). To cope with unfavourable copper concentrations, most bacteria utilize specific copper-induced defence mechanisms (RADEMACHER & MASEPOHL 2012). The chief mechanism of copper tolerance in bacteria is the active extrusion of copper from the cell (GRASS et al. 2011), and most bacteria, Gram-negative as well as Gram-positve, synthesize P-type copper export ATPases as principal defence determinants when copper concentrations exceed favourable levels (RADEMACHER & MASEPOHL 2012). However, bacteria also evolved a range of other mechanisms to protect themselves from the toxic effects of copper ions, such as extracellular sequestration of copper ions, relative impermeability of the outer and inner membranes to copper ions, and metallothionein-like copper-scavenging proteins in the cytoplasm and periplasm (GRASS et al. 2011). By copper-responsive gene regulation, bacteria can maintain cellular metabolism at different ambient copper concentrations (RADEMACHER & MASEPOHL 2012). These defence mechanisms, thus, might have contributed to the survival of a part of the population of the different target bacteria in a culturable state in copper containing ochre suspensions.

4.4.1.2 Acidic pH

Besides the toxic potential of different metal ions, another factor affecting hygienically relevant bacteria in ochre samples might be the local pH value. However, this might only be important in wells abstracting acidic water, and not in drinking water production wells, in which the pH is near neutral. The survival of *E. coli* in soil, for instance, was shown to be related to the local pH, and in particular soil acidity was detrimental (VAN ELSAS et al. 2011). For neutrophilic bacteria, which survive within the pH range of 5 to 8.5 and exhibit maximum growth rate at neutral or near neutral pH, low pH can be harmful. This is due to the denaturation of essential macromolecules, like proteins, and the acidification of the cytoplasm that disrupts enzymatic reactions and membrane potential (JEONG et al. 2008).

Such deleterious effect of low pH to cellular constituents could have caused the complete loss of culturability of *P. aeruginosa*, *L. pneumophila* and *A. hydrophila* in the microcosms containing the ochre sample VK 24 (pH 4.7), alongside with the high amount of copper, as discussed before. The more than 4-log reduction of culturable *A. hydrophila*, within the first four days of the experiment, was not that surprising since the observation that *Aeromonas* spp. are fairly sensitive to low pH (≤ 5.2) has been described in literature before (WANG & GU 2005). On the other hand, legionellae have been characterized as being relatively stable in acidic

conditions (BARTIE et al. 2003), which is why acid treatment (pH 2.2 5 min) is recommended for isolation of *Legionella* spp. from environmental water samples (BOPP et al. 1981), as well as for their detection and enumeration by the direct membrane filtration method for waters with low bacterial counts (ANONYMOUS 2008a), to minimize the growth of non-legionella-bacteria present in the samples. Hence, the decrease in numbers of CFU of more than 4 log units in ochre suspension VK 24 with a pH of 4.7, and of 2.3 log units in well water sample NW 83 with a pH of 3.4, for *L. pneumophila* was a bit surprising at first sight. However, the above mentioned acid treatment also decreases the number of legionellae recovered by cultivation from seeded water samples (BARTIE et al. 2001), which was also observed within the present study, as it eliminates not only non-legionella-bacteria but also part of the legionellae present in the treated samples (DITOMMASO et al. 2011). Some workers, therefore, consider acid treatment too drastic for legionellae; for example, in the Australian standard method, in contrast to the ISO method, acid treatment is only recommended for highly contaminated samples (BARTIE et al. 2003). Furthermore, concerning longer exposure of *Legionella* spp. to low-pH conditions, than the 5 minutes recommended for acid treatment, BOPP et al. (1981) reported that the plate count of *L. pneumophila* decreased about 1 log unit within 60 minutes of acid treatment (pH 2.2). Thus, the log reductions of *L. pneumophila* under acidic conditions, found in the present study, are quite comprehensible, since the exposure time was up to 14 days and additionally stressors, like metal ions and the indigenous microflora, were likely active in the ochre and well water microcosms.

Even though cell inactivation due to low pH seemed to play a role in the acidic samples in the microcosms, not only in ochre sample VK 24 but also in well water sample NW 83 (pH 3.4), in which *K. pneumoniae*, *E. coli*, and *L. pneumophila* showed the most pronounced decrease in numbers of CFU, compared to all other samples, not all target bacteria were equally sensitive. As described above, a complete loss of culturability was only detected for *P. aeruginosa*, *L. pneumophila* and *A. hydrophila* in VK 24 ochre microcosms, and in addition for *L. pneumophila* and *A. hydrophila* in well water NW 83 in the first run of the microcosm experiment. Furthermore, even though *L. pneumophila* in well water NW 83 was no longer culturable after day 7 (in the first run of the experiment), FISH-positive cells and genome units only decreased 0.3 and 0.5 log units, respectively. The bacteria, thus, still seem to be present but just no longer detected by the applied cultivation method. The significance of this finding is discussed below in section 4.4.4 about viable but non-culturable (VBNC) state bacteria.

The fact that not all target bacteria are completely inactivated under low pH conditions indicates that also neutrophilic bacteria possess acid resistance mechanisms. The primary strategy employed by bacteria to cope with acidic conditions is to maintain a constant cytoplasmic pH value, which is achieved by proton extrusion and cytoplasmic accumulation of potassium ions (KOBAYASHI et al. 2000). In enterococci, for instance, in acid medium, the cytoplasm is maintained at pH 7.6 by proton extrusion via the H^+-ATPase and K^+ accumulation, and membrane levels of ATPase increase in response to decreased cytoplasmic pH (KOBAYASHI et al. 2000).

The finding that *E. faecalis* survived best of all the target organisms in the acid microcosms (ochre VK 24 and water NW 83) is not surprising, since the species is described as a very stress-resistant bacterium in literature, for which extraordinary resistance to extreme pH values has been documented (AUFFRAY et al. 2011).

Moreover, acid resistance of enteric bacterial pathogens can also be enhanced by stresses experienced in aquatic environments, such as osmotic shock or nutrient starvation (GAUTHIER & CLÉMENT 1994). Acid resistance of *E. coli*, shigellae and *Salmonella typhimurium*, for instance, was enhanced by a hundred to a hundred million times after 100 minutes in seawater; an effect that was also observed in distilled water or phosphate buffers (GAUTHIER & CLÉMENT 1994). This effect could also have played a role in the microcosm experiments in the present study, since the target bacteria, in this case, were subjected to a similar stress by preparation of the inoculum in deionised water. Such an enhanced acid resistance, induced by oligotrophic waters, is of special concern, due to the fact that enteric bacterial pathogens entering water wells have, on the one hand, also experienced nutrient starvation and, on the other hand, have to cross the gastric barrier (pH 2.5 for about 2 h) to cause infection in humans.

Furthermore, different strains of the same species can show different capacities to survive in acidic environments, *E coli* O157:H7 strains, for example, all were superior in their survival over non-O157 EHEC (VAN ELSAS et al. 2011). Thus the response towards acidic conditions of the bacterial strains tested in this study might not be representative for other strains of the respective species.

4.4.2 Indigenous microbiota

Since allochthonous microbiota, entering water wells, are not going into a sterile microcosm but an environment already inhabited by native microorganisms, such organisms may play an important role in controlling the survival of non-native contaminant microbes (JOHN & ROSE 2005). For instance, the cumulative effect of the total indigenous microflora on *E. coli* surviv-

al is often negative as a result of predation, substrate competition and antagonism (VAN ELSAS et al. 2011). Because of this fact, the microcosm experiments were conducted with native, non-sterilised ochre and well water samples.

4.4.2.1 Heterotrophic plate counts of ochre and water samples

As a measure to characterise the ochre and well water samples microbiologically, the heterotrophic plate count (HPC) of the samples were determined at the beginning and the end of the microcosm experiments (d 0, d 14). Even though this method only detects the part of the indigenous microflora, which is culturable under the given conditions (cultivation on R2A medium at 20 °C for 7 days), the HPC is nonetheless useful to compare the different samples with one another, or with literature data.

The heterotrophic plate counts of the ochre samples in the present study (about $10^7 – 10^8$ CFU/g ochre dry mass) were in a similar range as reported for soft deposits removed from drinking water distribution networks, which were, like the ochre samples, mainly composed of iron (about $10^7 – 10^9$ CFU/g dry mass, ZACHEUS et al. 2001). So this seems to be a typical HPC value for iron containing deposits from oligotrophic water environments. However, not only the quantity of native heterotrophic microorganisms, present in an environment, influences the survival of allochthonous microbiota, but also the composition of the microbial community. For instance, the diversity of the indigenous microbial communities has been referred to as an important factor that regulates the population dynamics of invading *E. coli*, and, according to this view, ecosystems with a higher level of biodiversity are more resistant to perturbances than those with a lower diversity (VAN ELSAS et al. 2011). For ochreous deposits from drinking water and dewatering wells a high diversity of microorganisms has been found, and variation regarding the community structure was not only observed for different wells within one site but also for different deposit samples from one well (SCHRÖDER et al. 2014, WANG et al. 2014). These findings suggest that allochthonous microbiota have to cope with a lot of different microorganisms within the ochreous deposits of wells, and that the cumulative effect on their survival might be different even within one and the same well.

Even though the different ochre samples in the present study differed in their chemical composition and, according to the above findings of SCHRÖDER et al. and WANG et al. (2014), most likely also in their microbial composition, the HPC values were in a relatively narrow range of about $10^7 – 10^8$ CFU/g ochre dry mass and more or less constant over the course of the experiments. Thus, the seeded faecal indicator organisms and opportunistic pathogens

seem to have been exposed to similar amounts of native heterotrophic microorganisms in the ochre microcosms of the different samples, and the HPC cannot be used to explain differences in inactivation rates of the target organisms.

In the well water microcosms, this was different, since the plate counts of the water samples showed a wider range of about $10^2 – 10^6$ CFU/ml at day 0, and a more or less pronounced increase of up to 1.5 log units over the course of the experiments. So, the added target bacteria in these microcosms had to cope with unequal quantities of native heterotrophic microorganisms, and were therefore possibly subjected to different situations of competition and predation. However, the lowest plate counts of about 10^2 CFU/ml was found for the only acidic water sample (NW 83, pH 3.4) and, in the case of the water samples from dewatering wells, other parameters such as pH and origin of the samples seem to play a more significant role in controlling the survival of the target bacteria than the quantity of indigenous microorganisms. In contrast, for the water samples from drinking water wells, which were quite similar in pH and chemical composition, the higher amount of native microorganisms in water sample To-05 could have been responsible for the higher inactivation rates of *E. coli* and *E. faecalis* in this sample as compared to the ones in the other two samples from drinking water wells.

4.4.2.2 Well water samples: The "bottle effect" and high culturability

When comparing the HPC values of the well water samples of the present study with data from the literature, it is striking that the values found here, for the near neutral to slightly alkaline samples (about $10^4 – 10^6$ CFU/ml), are significantly higher than the ones reported for natural mineral waters (about 1 – 5 CFU/ml; LECLERC & MOREAU 2002) or for groundwaters ($10^1 – 10^2$ CFU/ml, 10^3 CFU/ml; STETZENBACH et al. 1986, LILLIS & BISSONETTE 2001). This is most likely due to bottling and storage of the samples. A rapid increase in numbers of viable counts after bottling of mineral water, attaining $10^4 – 10^5$ CFU/ml within 3 – 7 days, has been described in literature, as well as the so-called "bottle effect" or "volume effect" (LECLERC & MOREAU 2002). These terms were used to describe the observation that in stored water samples the number of bacteria was proportional to the surface area/volume ratio of the flasks or containers used for storage: The greater the surface area in relation to the volume of water was, the more rapidly growth of bacteria took place. The explanation for this is that nutrients, present in low concentration, are adsorbed and concentrated onto the surface, thereby being more available for the bacteria. Furthermore, in the case of groundwater, it is also possible that many of the more labile compounds, unavailable at the subsurface environments being complexed to lignins, phenolics, or adsorbed to clay minerals, become biodegradable

through sampling by interaction at the surfaces (LECLERC & MOREAU 2002). Therefore, the numbers of indigenous microorganisms in the water in the wells are likely lower than in the water microcosms, meaning that the negative impact of the native microflora on the target bacteria was probably higher in the microcosms than it would be in the wells.

Favourable nutrient conditions might also explain the high culturability of the heterotrophic microorganisms, in the near neutral to slightly alkaline well water samples, with ratios of heterotrophic plate counts to total cell counts of 22 – 65 %. Such a relationship has been described, for instance, for drinking water biofilms: High percentages of culturable cells in the range of 2 – 73 % on rubber-coated valves from drinking-water distribution systems (KILB et al. 2003) and of 15 – 18 % on plastic and elastomeric materials (MORITZ et al. 2010) were attributed to leaching of biodegradable organic compounds supplying additional nutrients to the biofilm organisms. In contrast, on inert metal, plastic or cement materials of drinking water pipes from different German drinking water distribution systems percentages of culturable HPC bacteria to total cell counts were in the range of 0.0004 – 3.5 % (WINGENDER & FLEMMING 2004).

Likewise, for oligotrophic to mesotrophic aquatic habitats it has frequently been reported that direct microscopic counts exceed plate counts by several orders of magnitude, with a culturability of e.g. 0.25 % for freshwater (AMANN et al. 1995). However, in an extensive study, examining more than 1000 samples from drinking-water systems all over Sweden, the percentage of culturable cells had a very high standard deviation and was dependent on the origin of the water sample: The fraction of culturable bacteria were of in the range of 0.0006 – 12 % in cold-water samples and of 0.015 – 58 % in warm water samples, where the culturability was always very low in samples from water mains and highest in hot water system and in-house installation samples (SZEWZYK et al. 2000). These findings are most likely attributed to favourable growth conditions for microorganisms in domestic plumbing systems as compared to those in water mains: (1) The inner diameter of pipes is much smaller, resulting in a larger ratio of surface to water volume, (2) longer periods of water stagnation may happen and water temperatures may increase due to insufficient insulation of the pipes, and (3) the choice of construction materials is less regulated than in the distribution system leading to a large variety of materials utilised, some of which might leach nutrients (MORITZ 2011). Thus, high fractions of culturable cells, on the one hand, seem to indicate good growth conditions, and, on the other, the absence of stress factors, which might reduce culturability by inducing the viable but non-culturable (VBNC) state (OLIVER 2005, 2010; LI et al. 2014, RAMAMURTHY et al.

2014). Low pH, as one of the VBNC state inducing factors (LI et al. 2014), conversely, could explain the much lower culturability, of only 0.7 – 4 %, in the acidic water sample NW 83 as compared to the near neutral to slightly alkaline well water samples.

4.4.3 Factors favouring the survival of hygienically relevant bacteria in ochre

In contrast to the possible stress factors in iron ochre, such as toxic metal concentrations or competition for nutrients and predation by the indigenous microbiota, ochreous deposits may also provide conditions favouring the survival of allochthonous microorganisms entering water wells. Particularly potential nutrient availability and the large surface area, as a substrate for bacterial attachment and the associated ecological advantages, should be beneficial for the persistence of hygienically relevant bacteria in ochre incrusted wells.

For chemoheterotrophs, such as the target bacteria, obtaining sufficient carbon compounds will clearly impact their chances of survival (VAN ELSAS et al. 2011, GORDON & TOZE 2003, HALLER et al. 2009), and organic matter contained in the ochreous deposits could represent such a source of nutrients in the otherwise oligotrophic environment of a groundwater well. For comparison, GAUTHIER et al. (1999) found organic matter, as well as bacterial biomass, in up to 2,000 times higher concentrations in deposits from a drinking water distribution system than in the circulating water and ZACHEUS et al. (2001), similarly, report that soft pipeline deposits were found to be the key site for microbial growth in drinking water distribution networks. Furthermore, the results of LEHTOLA et al. (2004) show that such soft deposits are able to release microbially available organic carbon and phosphorous into the bulk water when the water flow rate changed. Estimating the amount of organic matter in the ochre samples from the loss on ignition revealed that the samples contained about 11 – 22 % of organic carbon. However, higher ignition loss was not associated with clearly better survival of the target organisms in the respective sample and altogether the amount of organic carbon also did not give rise to growth of the target bacteria. Reasons for this might be that the organic carbon was either not bioavailable or not sufficient to promote bacterial growth, or that the opposing detrimental effects overcompensated beneficial effect of the nutrients.

Apart from the organic carbon content, the attachment to the large, porous surface of ochre incrustations in water wells might also favour the survival and persistence of hygienically relevant bacteria due to the integration into or formation of biofilms, which represent a protected mode of growth (HALL-STOODLEY et al. 2004). The biofilm matrix, i.e. the extracellular material, in which the biofilms cells are embedded and which is mostly produced by

the organisms themselves, confers tolerance to various antimicrobial agents, as well as protecting against some grazing protozoa (FLEMMING & WINGENDER 2010). Furthermore, the extracellular polymeric substances (EPS) keep the biofilm cells in close proximity, thereby allowing for intense interactions like the formation of synergistic microconsortia and the exchange of genetic information, and the EPS also retain enzymes enabling the digestion of macromolecules for nutrient acquisition (FLEMMING & WINGENDER 2010).

In general, the significance of the advantages of the biofilm mode of growth is reflected by the fact that most microorganisms on earth live in aggregates such as films, flocs, mats, granules or sludge (FLEMMING 2008). Concerning the persistence of hygienically relevant bacteria in the environment, in particular, the benefits of the presence of particulate surfaces to be colonised has been shown in different laboratory survival experiments. *E. coli* (BURTON et al. 1987, GARZIO-HADZICK et al. 2010, KORAJKIC et al. 2013), fecal coliforms and fecal streptococci (SHERER et al. 1992), and also *P. aeruginosa* and *K. pneumoniae* (BURTON et al. 1987) all survived significantly longer in sediments or sediment laden waters than in the overlying waters or water without sediment. In the study of BURTON et al. (1987) *P. aeruginosa* and *K. pneumoniae* tended to survive longer than *E. coli*, just like in the present study. This, however, is not that surprising, since both *P. aeruginosa* and *Klebsiella* spp. are common environmental organisms, whereas *E. coli* normally lives in the intestinal system of mammals.

In groundwater microcosms, T_{90} values (the time taken for 90 % die-off, i.e. 1 log reduction) for *E. coli* were 7 ± 3 d (BANNING et al. 2002; anaerobic conditions in the dark at 28 °C) and 6 d (BITTON et al. 1983; in the dark at 22 °C), respectively, as compared to 7 ± 4 d in the present study (in the dark at 17 °C). Concerning the survival experiments in ochre suspension, conducted in the present study, no literature data for comparison was found. However, even though the survival of hygienically relevant bacteria was not, in general, enhanced in ochre suspension, as compared to well water, most target bacteria survived significantly longer attached to ochre under flow-through conditions than in well water microcosms. This is in accordance with SHERER et al. (1992) suggesting that sediment allows enteric bacteria to survive for months in aquatic environment rather than the days as typically measured in water.

4.4.4 The viable but non-culturable (VBNC) state in bacteria

Another factor maybe favouring the survival of hygienically relevant bacteria in ochre, is the entry into the viable but non-culturable (VBNC) state, a unique survival strategy adopted by many bacteria in response to adverse environmental conditions (OLIVER 2010, LI et al. 2014,

RAMAMURTHY et al. 2014; cf. page 55). This state can be thought of as an inactive form of life waiting for revival under suitable conditions (RAMAMURTHY et al. 2014). Since, the target organisms in the present study are all known to enter the VBNC state (OLIVER 2005, 2010), culture-independent methods, FISH and qPCR, were applied alongside the traditional cultivation techniques to quantify the target cells present in a sample.

However, in ochre suspensions, unfortunately, no quantification of target organisms by FISH could be achieved, since the ochre particles interfered significantly with the fluorescence microscopic quantification of the cells. This interference also made staining procedures, used as viability markers, such as the LIVE/DEAD® BacLight™ assay or direct viable count (DVC-FISH) (LI et al. 2014, RAMAMURTHY et al. 2014), not applicable. Thus, qPCR as an alternative cultivation-independent method was used, even though this method has its limitations as well. The major drawback of the qPCR methods applied is that it quantifies target bacteria, *L. pneumophila* or *P. aeruginosa*, irrespective of their physiological state, as long as the genomic sequence, which is targeted, is intact. Hence, no statement can be made concerning the viability of the target bacteria detected by qPCR.

More recently, pre-treatments with ethidium monoazide (EMA) or propidium monoazide (PMA) prior to DNA extraction, have been proposed to circumvent this drawback by inhibiting the amplification of DNA which is not protected by an intact cell membrane (WHILEY & TAYLOR 2014). In theory, the DNA-intercalating agents EMA and PMA only penetrate cells with damaged membranes where they bind to DNA, when exposed to light, thereby preventing the amplification and quantification of DNA from cells with non-intact membranes in the subsequent qPCR run (WHILEY & TAYLOR 2014, BAE & WUERTZ 2009). However, these methods have not been optimised for differing sample types and their reliability and the accuracy of the results is still debated (WHILEY & TAYLOR 2014, TAYLOR et al. 2014). Besides, solid concentrations of 1 mg/ml have been reported to affect the suppression of PCR amplification from heat-killed cells by interfering with the cross-linking of PMA (BAE & WUERTZ 2009). Since the ochre suspensions contained about 100 mg ochre wet mass/ml, pre-treatment with EMA or PMA seemed not applicable.

The quantification of target organisms by FISH, which was possible in well water samples, however, could possibly give an indication of the physiological state of the bacteria, since a strong fluorescent signal reflects a high ribosome content, which indicates an elevated metabolic activity of the thus detected cells (GRIMM et al. 1998, MOTER & GÖBEL 2000, AMANN & FUCHS 2008). Though, attempts to use rRNA as a viability marker have yielded inconsistent

results, for instance due to the high stability of rRNA after killing of cells, or because different stress conditions applied to cells subsequently resulted in different contents of rRNA (LAHTINEN et al. 2008, SHERIDAN et al. 1998, TOLKER-NIELSEN et al. 1997). Hence, a FISH positive signal should only be assessed as an indication of bacteria in the VBNC state. Thus, the higher numbers of FISH-positive cells, as compared to the numbers of colony forming units, in the spiked as well as in the native well water samples might be explained by target bacteria having entered the VBNC state.

For qPCR, similarly, also higher numbers of genome units (GU) than colony counts had been expected. However, this was not true for all samples and mainly just for *L. pneumophila* qPCR, and the difference between the qPCR results and the results gained by cultivation was not as high as might have been expected. The *P. aeruginosa* qPCR assay even yielded lower GU numbers than colony counts for the majority of samples. This might be due to an inhibition of the qPCR by matrix compounds (WHILEY & TAYLOR 2014), since such contaminants may interfere with the reaction, for instance, high concentrations of iron and manganese (WULLINGS & VAN DER KOOIJ 2006). As the *P. aeruginosa* qPCR assay did not comprise an internal control, a possible inhibition of the qPCR reaction cannot be proven. Another reason for the low numbers of GU, especially gained for the well water samples, might be the low DNA yield obtained for the DNA extraction of the *P. aeruginosa* spiked water samples. The extraction yielded only 16 to 143 ng per preparation, for the spiked water samples of day 0 of the microcosm experiments, as compared to 60 to 1416 ng per preparation for the water samples spiked with *L. pneumophila*. The DNA yield for the ochre samples, for comparison, was 190 to 333 ng per preparation for the *P. aeruginosa* spiked samples and 198 to 276 ng per preparation in the case of *L. pneumophila* spiked samples. Altogether, the inconsistency of the results show the need for further validation and adjustment of the qPCR methods for ochre and well water samples.

4.5 Colonisation of ochre by hygienically relevant bacteria and contamination of the water phase

Wells abstracting anaerobic groundwater with high amounts of reduced iron and manganese species are prone to the formation of ochreous deposits in or even around the well, and also in the pump and rising pipe. The incrustations, which are formed, result from the oxidation of the water-soluble reduced forms of the metals yielding water-insoluble products. This process of well clogging, which can proceed abiotically when the anoxic groundwater gets in contact

with oxygen or can be mediated by microorganisms (e.g. iron-depositing bacteria), leads to the formation of very large porous surfaces in water wells.

"The proclivity of bacteria to adhere to surfaces" (HALL-STOODLEY et al. 2004) led to the question if hygienically relevant bacteria, introduced into a water well by some kind of contamination event, can colonise the ochre surface and be released from it into the water phase later again. Such a contamination of a water well was simulated in column experiments. The results of those experiments (section 3.6) show that faecal indicator bacteria and opportunistic pathogens, brought in contact with ochre, attach to the ochre surface. All six species of target bacteria, injected into ochre containing columns and left stagnant in the columns for 17 h (5 h in the case of *L. pneumophila*), could be recovered from the ochre. Hence, the attachment of the bacteria to the ochre, as well as to other surfaces of the system, was proven.

It could also be shown that a release of target bacteria from out of the ochre takes place over the whole time period of the experiments (14 days). The mechanism of detachment and release could be active as well as passive dispersal from the ochre, similar to the dispersal of cells from a biofilm (see Figure 52). Here, the production of specific dispersal cells at the final stage of the biofilm life cycle is an active and highly regulated response, in contrast to the passive dispersal resulting from sloughing of cells and erosion from the biofilm caused by external perturbations, such as increased fluid shear (HALL-STOODLEY et al. 2004, MCDOUGALD et al. 2011).

The problem of biofilms being the source of water contamination by coliforms has been described, for instance, by KILB et al (2003) for rubber-coated valves from drinking water distribution systems. Coliform bacteria were found over a period of several months in water samples taken at hydrants located in proximity to rubber-coated valves. An inspection of the valves revealed biofilms containing the same species of coliforms as identified in the drinking water samples. This example demonstrates the prolonged water contamination potential of biofilms containing hygienically relevant bacteria.

A rough calculation for the flow-through experiments with external inoculation of the ochre (results given in section 3.6.2) suggests the same. A continuous release of hygienically relevant bacteria from out of the ochre into the water phase could theoretically happen for up to months to two years, considering the amount of target bacteria detected in the ochre and the amount washed out of the columns after 1 h, if the wash out of target bacteria happens to be linear and disregarding their die-off or growth. Even though this calculation is just a thought

experiment, it gives a hint how long lasting a water contamination might be, if ochre is polluted by hygienically relevant bacteria.

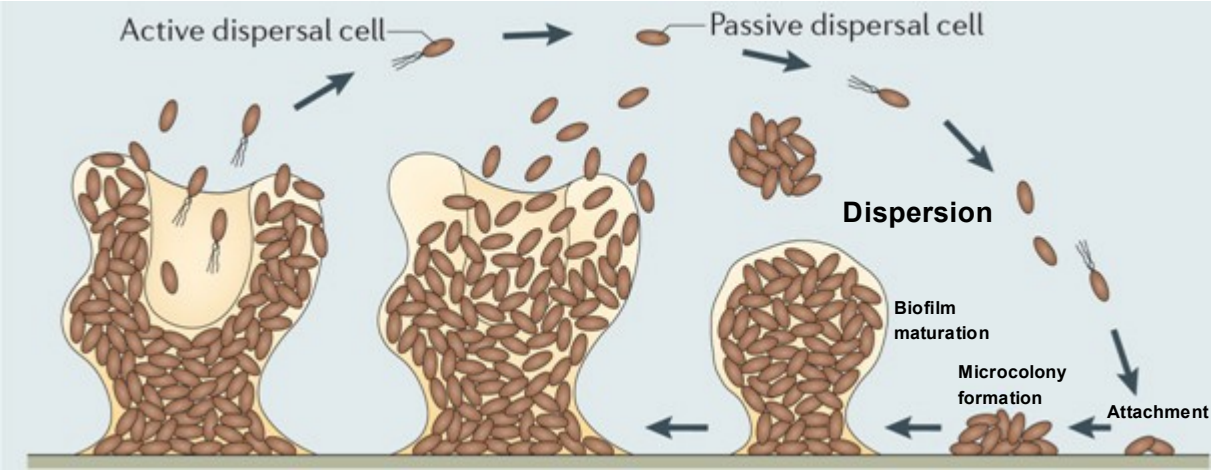

Figure 52: Biofilm life cycle and dispersal. The biofilm life cycle is characterised by an orderly and predictable transition through a series of stages, each with a set of genes that is uniquely expressed during that stage, leading to particular phenotypes and culminating in a dispersal event. The biofilm life cycle consists of active dispersal (triggered by signals such as starvation, rhamnolipids, phages, and cell death) and passive dispersal (as occurs through streamers, rolling dispersal and sloughing), both of which result in the release of planktonic cells that undergo attachment and biofilm maturation to form microcolonies. Environmental cues (e.g. low nutrient conditions) can induce transition between, or initiation of, these different stages in the biofilm life cycle (After McDOUGALD et al. 2011, modified).

However, the persistence of such bacteria attached to ochre, for at least weeks, has been proven by the flow-through experiments, which resulted in rather low log reductions of the target bacteria over the course of the experiments (14 d). *P. aeruginosa* and *L. pneumophila* showed no or nearly no reduction; the maximum reduction of about one log unit was observed for *E. coli*. Such a persistent contamination of the ochre, by indicator bacteria and pathogens, in a drinking water well, therefore, could degrade the hygienic quality of the raw water repeatedly and long-lasting, posing a significant threat to public health, if the groundwater source is used for public supply only with a minimum level of treatment (e.g. chlorination), or with no treatment at all. This is, in particular, a matter of concern, since a disinfection of such a contaminated well, using hydrogen peroxide, might not be effective against hygienically relevant bacteria attached to ochre, as discussed in the following section.

4.6 Disinfection experiments using H_2O_2

For disinfection of wells, e.g. in case the bacteriological examination of the raw water, after a well rehabilitation, yields a result exceeding the limit values of the German Drinking Water Ordinance, or for a regular treatment to decelerate the biotic formation of ochreous deposits in

wells, hydrogen peroxide is recommended to be used with an application concentration of about 150 mg/l (ANONYMOUS 2001b, ANONYMOUS 2007). Concerning periodic disinfection, the Berliner Wasserbetriebe, for instance, introduce hydrogen peroxide solutions of 1-2 % into the wells to retard well ageing. Depending on the application method, this yields a target concentration of hydrogen peroxide in the well of about 0.3 g/l (if evenly distributed within the well) or even of 10 g/l (if only added at one spot just above the upper edge of the filter).

Such hydrogen peroxide concentrations were tested against hygienically relevant bacteria (*E. coli*, *E. faecalis* or *P. aeruginosa*) attached to ochre, to estimate the efficacy of a well disinfection after a contamination event, introducing faecal indicator bacteria or pathogens into existing ochre incrustations, has happened. The experiments showed that the hydrogen peroxide concentration recommended for well disinfection, about 150 mg/l, was ineffective against hygienically relevant bacteria attached to ochre; even the addition of a single dose of a more than 200 times higher concentrated H_2O_2 solution (up to 34 g/l; contact time: 24 h) resulted only in a minor decline in culturability of about 0.4 to 0.9 log units of the respective bacteria. Only when hydrogen peroxide was added continuously over a time period of 24 h, an appreciable reduction in numbers of culturable target bacteria could be achieved.

This enormous resistance is quite striking, since normally oxidative stress should arise when ferrous (Fe^{2+}) or ferric iron (Fe^{3+}) react with hydrogen peroxide to produce harmful ROS, which cause cellular damage as a result of their high reactivity. This indicates that the mechanisms, described in detail in section 4.3.1 (regarding the question if target bacteria are inactivated in contact with ochre due to oxidative stress),

- the tight control of iron uptake and storage in bacteria (see section 4.3.1.1)
- the many enzymes that scavenge intracellular hydrogen peroxide (see section 4.3.1.2
- if cellular damage by ROS has occurred, the mechanisms to repair it (see section 4.3.1.3)
- and the catalytic decomposition of exogenous hydrogen peroxide on the surface of the iron oxide ochre (see section 4.3.1.4)

are also sufficient to protect the target bacteria even in the case of high amounts of exogenous hydrogen peroxide being present.

The significance of the last point, the rapid decomposition of hydrogen peroxide in the presence of ochre, could clearly be demonstrated in the present study. After 15 minutes contact time, ochre suspensions, mixed with hydrogen peroxide solutions, contained only about 25-50 % of the initial H_2O_2 concentration of 10 g/l. And also the influence of the bacteria itself

on the stability of H_2O_2 was observed, since in the preparations containing *P. aeruginosa* a more pronounced decrease of the hydrogen peroxide concentration over time was detected as compared to the preparations containing *E. coli* or *E. faecalis*. These findings suggest that *P. aeruginosa* produces and secretes hydrogen peroxide scavenging enzymes which even can decompose elevated amounts of exogenous H_2O_2.

The result that hydrogen peroxide is ineffective against hygienically relevant bacteria attached to ochre, clearly emphasises to basic necessities:

1. The need to protect drinking water wells from external contamination, and
2. the imperative of thorough cleaning of ochre incrusted wells before disinfection.

4.7 Conclusion

The results of the present work show, in the first place, that all six bacterial strains, selected as examples of hygienically relevant bacteria, attached to the surface of iron ochre, sampled from water wells, and, secondly, were not completely inactivated due to oxidative stress when brought in contact with the ochre.

Even though no growth of the bacteria was detected when added into ochre, still a part of the population of each target bacterium survived, even in a culturable state, up to 14 days in ochre suspensions in microcosms, as well as attached to ochre under flow-through conditions (both at 17 °C in the dark). In the latter case, the survival was even better than in the batch preparations of the ochre suspensions. The lowest log reduction after 14 days, of 0.1 and 0.4, or even no reduction at all was detected for the environmental bacteria and opportunistic pathogens, *Aeromonas hydrophila*, *Legionella pneumophila* and *Pseudomonas aeruginosa*, attached to ochre under flow-through of synthetic groundwater. In contrast, the coliform bacterium, *Klebsiella pneumoniae*, opportunistic pathogen as well as indicator of a general water contamination, and the gram-positive bacterium, *Enterococcus faecalis*, indicator of faecal water pollution, both showed a decrease of 0.8 log units. The most pronounced reduction of 1.1 log units after 14 days was observed for the faecal indicator bacterium, *Escherichia coli*.

A contamination of the water, in contact with the ochre, by the hygienically relevant bacteria from out of the ochre, was proven for all six bacterial strains over the whole period of the experiments (14 days). Considering the persistence of the hygienically relevant bacteria in the ochre, as outlined above, and their wash out from the ochre, detected in the flow-through experiments, a water contamination by those bacteria could theoretically last for months to two

years. Such a persistent contamination of the ochre, by indicator bacteria and pathogens, in a drinking water well, therefore, could degrade the hygienic quality of the raw water repeatedly and long-lasting, posing a significant threat to public health, if the groundwater source is used for public supply only with a minimum level of treatment (e.g. chlorination), or with no treatment at all.

The recommended disinfection, using hydrogen peroxide solution of a concentration of about 150 mg/l, was ineffective against hygienically relevant bacteria (*E. coli*, *E. faecalis* or *P. aeruginosa*) attached to ochre. Even the addition of a more than 200 times higher concentrated H_2O_2 solution (up to 34 g/l; contact time: 24 h) resulted only in a minor decline in culturability of about 0.4 to 0.9 log units of the respective bacteria.

In the presence of ochre, hydrogen peroxide was rapidly decomposed, and also the presence of bacteria (*P. aeruginosa*) caused a decrease of the H_2O_2 concentration; most probably due to the production and secretion of high amounts of hydrogen peroxide scavenging enzymes.

Altogether, these results demonstrate that ochreous incrustations in drinking water wells can function as sink and source for hygienically relevant bacteria and pathogens, if such microorganisms are introduced into the wells by some kind of contamination event. The ineffectiveness of hydrogen peroxide solution (up to 3 % (v/v) application concentration, corresponding to about 34 g/l) against hygienically relevant bacteria attached to ochre, underscores the basic necessity to protect groundwater and drinking water wells from external contamination, in order to prevent the ingress of pathogens and, thus, their integration into ochreous deposits in the wells. Furthermore, it makes clear that thorough cleaning of an ochre incrusted and microbially polluted well before a hydrogen peroxide treatment is imperative for disinfection. The disinfection efficacy of such a treatment of wells in practice, however, is doubtful, since a complete removal of all deposits from a well, especially those outside the annulus, which are practically out of reach for all current well rehabilitation techniques (HOUBEN & WEIHE 2010), is unlikely to be achieved. Therefore, further research is needed, especially to develop strategies to prevent the formation of incrustations in wells, or to dissolve and remove it. Moreover, the development of culture-independent methods, able to quantify all viable microorganisms or the viable fraction of particular target species, e.g. of hygienic relevance or of relevance in ochre formation, is necessary in order to assess the microbial status of a well or to detect a contamination event.

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6 Annex

6.1 Well rehabilitation

Table 30: Mechanical processes for well rehabilitation, Part 1 (Anonymous 2007).

	Cleaning		Intensive abstraction	Piston	CO_2 injection	Low-pressure interior flushing
	Brushes	**Pumping-out/ well sump cleaning**				
Scope of application	- slightly solidified deposits	- deposition in the well pipe - smaller foreign objects	- slightly solidified deposits	- slightly to medium solidified deposits	- slightly to highly solidified deposits	- slightly solidified deposits
Method of operation	- detach deposits by moving up and down and/or by rotating circular brushes	- pumping out e.g. with an airlift pump, if necessary with simultaneous mixing up of depositions	- step-by-step and intermittent pumping out - optimum effect by moving up and down in the section	- generation of a suction effect with valve pistons by up-and-down movement of the tool - treatment of the filter in appropriate sections of up to 5 m	- after replacing the water in the well by gaseous CO_2, liquid CO_2 is injected to freeze the deposits ("frost bursting")	- low pressure jets of water are aimed at the pipe wall - operation as circulating system because of high water demand
Operating range	- pipe wall, partially screen slots	- well sump or rather lower part of the well	- screen slots, gravel pack	- screen slots, gravel pack and adjacent formation	- screen slots, gravel pack and adjacent formation	- screen slots, gravel pack
Progress check	- checking of solid matter content by continuous concurrent pumping out	- continuous measuring of the height of depositions and monitoring of the solid matter content	- checking of solid matter content by continuous concurrent pumping out	- checking of solid matter content by continuous concurrent pumping out	- not possible	- checking of solid matter content by continuous concurrent pumping out
Termination	- decrease in solid matter content	- reaching the well floor - no solid matter in the discharge volume	- decrease in solid matter content - aquifer stabilised to prevent sand pumping	- decrease in solid matter content	- constant CO_2 influx at a consistent pressure	- decrease in solid matter content
Stop criterion	- extraction of parts of the well construction material and the gravel pack	- extraction of parts of the well construction material and the gravel pack	- increased sand pumping - spontaneous increase in solid matter content -- extraction of parts of the well construction material and the gravel pack	- extraction of parts of the well construction material and the gravel pack - spontaneous increase in deposition	- no pressure build-up possible - pressure drop below 5 bar - uncontrollable icing of the System or rather in the well	- intense, spontaneous increase in deposition - extraction of parts of the well construction material and the gravel pack
Load of the construction material	- low when operated on a rope - demanding when using a rod system and rotating operation	- low	- low to medium	- medium to high	- medium to high	- low

Table 31: Mechanical processes for well rehabilitation, Part 2 (Anonymous 2007).

	High-pressure flushing procedures		Shock wave-/Pulse methods – generation by			
	Internal flushing	**External flushing**	**High pressurized water**	**Oxyhydrogen gas, water- or air compression**	**Explosive charge**	**Ultrasound**
Scope of application	- slightly to highly solidified deposits	- slightly to medium solidified deposits	- slightly to highly solidified deposits	- slightly to highly solidified deposits	- slightly to highly solidified deposits	- slightly to medium solidified deposits
Method of operation	- high pressure water jet aimed at the pipe wall by moving up and down rotating injectors with continuous concurrent pumping out	- water jetting of lances equipped with injectors into the gravel pack - direct high-pressure flushing of the filter gravel	- vibrations are generated by very fast rotating injectors and very high water pressure which are transmitted over the pipe wall to the gravel pack and the outer field; continuous concurrent pumping out	- adjustable pressure pulses released to the pipe wall, gravel pack and the surrounding area by e.g. Oxyhydrogen gas, water- or air compression; continuous concurrent pumping out	- generation of an impulse by ignition of a detonating cord in the filter pipe with subsequent compression-decompression of the gas bubble - treatment of the whole filter section in one operation possible	- generation of over- and underpressure in steady alternation by ultra-sound - step-by-step treatment of the filter sections top down
Operating range	- pipe wall, screen slots, gravel pack	- gravel pack	- pipe wall, screen slots, gravel pack and adjacent formation	- pipe wall, screen slots, gravel pack and adjacent formation	- screen slots, gravel pack and adjacent formation	- gravel pack
Progress check	- checking of solid matter content by continuous concurrent pumping out	- concurrent pumping out in the well	- checking of solid matter content by continuous concurrent pumping out	- checking of solid matter content by continuous concurrent pumping out	- indirectly by additional, step-by-step controlled intensive abstraction	- alternately ultrasound and subsequent pumping out of the same section of the filter
Termination	- decrease in solid matter content	- decrease in solid matter content	- decrease in solid matter content	- decrease in solid matter content	- decrease in solid matter content	- decrease in solid matter content
Stop criterion	- extraction of parts of the well construction material and the gravel pack	- extraction of parts of the well construction material, the gravel pack and the adjacent formation	- extraction of parts of the well construction material and the gravel pack	- extraction of parts of the well construction material and the gravel pack	- not definable	- extraction of parts of the well construction material and the gravel pack
Load of the construction mate-	- medium to very high	- very high	- medium to high	- medium to high	- high to very high	- very low

Table 32: Chemical processes for well rehabilitation (Anonymous 2007).

	Multiple chamber devices
Scope of application	• medium to highly solidified deposits
Method of operation	• circulation of the rehabilitation agent in sections - circulation control adapted to the well geometry and the radial depth of penetration - with or without reversible direction of flow • High amounts of deposits: intermediate pumping out of saturated solutions, if necessary with monitoring and repetition of the dissolving processes
Operating range	• screen slots, gravel pack and adjacent formation
Dosage control to check application concentration	• acids: pH value • other rehabilitation agents: according to action principle and with appropriate parameters
Progress check	• comparison of time with a dissolution test • measuring of ion concentration (for evaluation of the dissolution kinetics, determination of the discharged amount as needed) • Measurement of turbidity
Loss of rehabilitation agents	• low to medium (device-dependent)
Termination	• decrease in dissolving effect
Stop criterion	• rehabilitation agent consumption above-average • insufficient recovery of the rehabilitation agent during intermediate pumping out
Load of the construction material (device-dependent)	• low
Load of the construction material (rehabilitation agent-dependent)	• low to high

6.2 Standard deviations of the mean values given in the figures in section 3.5.3

Table 33: Standard deviations of mean values (shown in Figure 21) of two consecutive runs of microcosm experiments with *E. coli* or *E. faecalis* spiked in suspensions ochre samples (O.) and water samples (W.) from dewatering wells. In bold: Standard deviations of ≥ 1 log unit. - = no mean value determined.

		Standard deviation			
		Sampling day			
Species	**Sample**	**4**	**7**	**11**	**14**
E. coli	O. H 1424	0.42	0.30	0.31	0.91
	O. W 5465	0.43	0.49	0.36	0.11
	O. NW 83	0.10	0.12	0.72	0.68
	W. H 1424	-	**1.72**	**1.01**	0.83
	W. W 5465	0.18	-	0.12	0.13
	W. NW 83	-	**1.37**	**1.11**	**1.11**
E. faecalis	O. H 1424	0.10	0.09	0.05	0.08
	O. W 5465	0.50	0.47	0.37	0.40
	O. NW 83	0.05	0.07	0.13	0.13
	W. H 1424	0.17	0.18	0.08	0.40
	W. W 5465	0.06	0.58	0.14	0.42
	W. NW 83	0.15	0.47	0.06	0.17

Table 34: Standard deviations of mean values (shown in Figure 22) of two consecutive runs of microcosm experiments with *P.aeruginosa* or *L. pneumophila* spiked in suspensions of ochre samples (O.) and in water samples (W.) from dewatering wells. In bold: Standard deviations of ≥ 1 log unit. - = no mean value determined.

		Standard deviation Sampling day			
Species	**Sample**	**4**	**7**	**11**	**14**
P. aeruginosa	O. H 1424	0.81	0.17	0.03	0.12
	O. W 5465	**1.31**	0.40	0.30	0.11
	O. NW 83	0.25	0.50	0.06	0.26
	W. H 1424	0.50	0.31	0.16	0.22
	W. W 5465	0.46	0.20	0.45	0.64
	W. NW 83	-	0.58	0.34	-
L. pneumophila	O. H 1424	0.90	0.31	0.63	0.90
	O. W 5465	0.03	0.14	0.14	0.08
	O. NW 83	0.05	0.30	0.23	0.38
	W. H 1424	0.67	0.35	0.26	0.23
	W. W 5465	0.12	0.17	0.07	0.01
	W. NW 83	0.04	0.22	-	-

Table 35: Standard deviations of mean values (shown in Figure 23) of two consecutive runs of microcosm experiments with *K. pneumoniae* or *A. hydrophila* spiked in suspensions of ochre samples (O.) and in water samples (W.) from dewatering wells. In bold: Standard deviations of ≥ 1 log unit. - = no mean value determined.

		Standard deviation Sampling day			
Species	**Sample**	**4**	**7**	**11**	**14**
K. pneumoniae	O. H 1424	0.28	0.35	0.84	0.73
	O. W 5465	0.01	0.02	0.08	-
	O. NW 83	0.26	0.07	0.54	0.61
	W. H 1424	0.88	**1.00**	**1.24**	**1.24**
	W. W 5465	0.15	0.86	0.28	0.16
	W. NW 83	0.51	0.95	0.46	0.74
A. hydrophila	O. H 1424	0.24	0.45	0.29	0.88
	O. W 5465	0.08	0.11	0.10	0.09
	O. NW 83	0.41	0.33	0.13	0.14
	W. H 1424	0.44	0.13	0.29	0.53
	W. W 5465	0.08	0.04	0.49	0.12
	W. NW 83	-	-	-	-

Table 36: Standard deviations of mean values (shown in Figure 24) of two consecutive runs of microcosm experiments with *E. coli* or *E. faecalis* spiked in suspensions of ochre samples (O.) and in water samples (W.) from drinking water wells. In bold: Standard deviations of ≥ 1 log unit. - = no mean value determined.

		Standard deviation Sampling day			
Species	**Sample**	**4**	**7**	**11**	**14**
E. coli	O. Tw-21	-	0.03	-	0.35
	O. To-05	0.17	0.03	0.03	0.00
	O. FRI-25	0.33	0.05	0.22	0.36
	W. Tw-21	0.07	0.10	0.57	0.14
	W. To-05	0.11	0.23	0.31	0.26
	W. FRI-25	0.11	0.07	0.21	0.03
E. faecalis	O. Tw-21	-	0.01	-	0.07
	O. To-05	0.01	0.90	0.90	**1.10**
	O. FRI-25	0.30	0.05	0.12	0.15
	W. Tw-21	0.05	0.07	0.02	0.09
	W. To-05	0.08	0.28	0.13	0.16
	W. FRI-25	0.46	0.26	0.29	0.26

Table 37: Standard deviations of mean values (shown in Figure 25) of two consecutive runs of microcosm experiments with *P. aeruginosa* or *L. pneumophila* spiked in suspensions of ochre samples (O.) and in water samples (W.) from dewatering wells. In bold: Standard deviation of about 1 log unit. - = no mean value determined.

		Standard deviation Sampling day			
Species	**Sample**	**4**	**7**	**11**	**14**
P. aeruginosa	O. Tw-21	-	0.34	-	0.13
	O. To-05	0.13	0.34	0.23	0.35
	O. FRI-25	0.79	0.58	0.69	**0.98**
	W. Tw-21	0.48	0.23	0.14	0.24
	W. To-05	0.19	0.27	0.09	0.30
	W. FRI-25	0.64	0.43	0.71	0.00
L. pneumophila	O. Tw-21	-	0.05	-	0.57
	O. To-05	0.10	0.16	0.18	0.27
	O. FRI-25	0.21	0.36	0.07	0.31
	W. Tw-21	0.21	0.22	0.05	0.40
	W. To-05	0.14	0.07	0.20	0.00
	W. FRI-25	0.15	0.01	0.14	0.22

Table 38: Standard deviations of mean values (shown in Figure 26) of two consecutive runs of microcosm experiments with *K. pneumoniae* or *A. hydrophila* spiked in suspensions of ochre samples (O.) and in water samples (W.) from drinking water wells. Standard deviations in log units. - = no mean value determined.

		Standard deviation Sampling day			
Species	**Sample**	**4**	**7**	**11**	**14**
K. pneumoniae	O. Tw-21	-	0.09	-	0.08
	O. To-05	0.24	0.53	0.50	0.43
	O. FRI-25	0.03	0.14	0.34	0.25
	W. Tw-21	0.63	0.04	0.06	0.03
	W. To-05	0.27	0.19	0.14	0.05
	W. FRI-25	0.02	0.52	0.53	0.14
A. hydrophila	O. Tw-21	-	0.19	-	0.35
	O. To-05	0.15	0.04	0.36	0.12
	O. FRI-25	0.15	0.15	0.16	0.24
	W. Tw-21	0.06	0.19	0.16	0.52
	W. To-05	0.42	0.40	0.32	0.41
	W. FRI-25	0.32	0.18	0.58	0.28

Publikationsliste

Publikationen

Mattes, H., Alfer, B., Shokhrin, V. (2010): Bird migration in autumn 2005 at the coast of the Japanese Sea, Lazovsky Zapovednik, Primorye. In: Mattes, H. (Hrgs.): Living alongside the Tiger – The Fauna of the Lazovsky Zapovednik, Sikhote Alin. Arb. Inst. Landschaftsökol. Münster 18: S. 59-71 – ISSN 1431-1313.

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Vortrag

Alfer, B., Michalowski, W. D., Flemming, H.-C., Wingender, J. (2011). Dynamics of microbial populations and biochemical composition of drinking-water biofilms. Vortrag und Beitrag im Tagungsband der IWA Biofilms Conference 2011 - Processes in Biofilms, Shanghai/China.

Poster

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Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Integration, persistence and control of hygienically relevant bacteria in iron oxide incrustations in wells"

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im Juli 2015

(Barbara Dericks)

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